CANADA

VERIFICATION:

DEVELOPMENT OF

A PORTABLE TRICHOTHECENE SENSOR KIT

FOR THE DETECTION OF

T-2 MYCOTOXIN IN HUMAN BLOOD SAMPLES

MARCH 1987

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PREFACE

The seriousness of an allegation of the use of chemical weapons cannot be overstated. Since the early-1980s, the international community has had to grapple with the procedural and organizational problems relating to the initiation of investigations of such allegations. In this regard, the United Nations General Assembly and, in particular, the United Nations Secretary-General have had to address especially sensitive issues in the absence of a well-defined treaty framework to pursue these matters. The Canadian Government has been profoundly disturbed by allegations of the use of chemical weapons in various regions of the world and has fully supported efforts attempting to confirm or refute the allegations.

In addition to the procedural and organizational problems mentioned above, there are also operational and technical problems which complicate efforts to confirm or refute an allegation. Some of these operational problems have been identified and addressed in the Handbook for the Investigation of Allegations of the Use of Chemical or Biological Weapons, which was presented by Canada to the United Nations Secretary-General in December 1985.

The speedy collection and subsequent analysis of samples pose many problems to an investigating team. These include having to make decisions about what samples to collect and in what numbers, especially since samples would then have to be sent to sophisticated laboratories for further analysis. These difficulties are compounded many times if the team is investigating an incident involving an allegation of the use of a "novel" agent, that is, of a chemical substance not previously used for or associated with hostile purposes. Additional complications arise in the event that such substances also occur naturally in the environment, whereas a different set of problems would derive from the use of a (previously) totally unknown substance. In other words, investigations may be far from straightforward affairs and, as was mentioned in the Handbook, may amount to a scientific expedition carried out under stressful and possibly dangerous conditions.

The Canadian Government, through the Verification Research Unit of Canada's Department of External Affairs, was interested in developing a better understanding of the kind of scientific base that would have to be mobilized in order to support a field investigation involving an allegation of the use of a novel agent. Trichothecene mycotoxins provided suitable material for such a study. Some of the research has

been directed at the development of simple techniques for sample collection, packaging, recording and transportation; and at the development of more precise techniques for analysis in "rear area" laboratories. These will be the subject of a separate report.

The present report provides details of a study commissioned by the Canadian Government, and executed by an internationally-known biotechnology institute, to develop a light-weight easily-transportable kit for use in the field as a screening assay in the detection, identification and quantification of T-2 mycotoxin in human blood samples. This research project was undertaken as a case study, to develop a better understanding of the technical problems associated with the provision of appropriate sensors to an investigating team.

Work began on this project in late-1984 and continued through the final report-writing phase which was completed in December 1986. It involved contributions at various times from a half-dozen scientists and from a similar number of technical support personnel. The report outlines the initial goals, problems encountered and successes achieved, and the Institute's recommendations of additional work to take the sensor kit from its present stage as a prototype to the final stage of a fully-operational kit.

As a case study, we believe this report will be of interest to scientists and organizations that must look beyond the headlines and deal with the very real, very demanding and very time-consuming technical problems related to verification. We consider it relevant not only in the context of work being conducted within the current international legal framework, but also to work which will need to be performed within the framework of an eventual comprehensive convention banning chemical weapons. In the latter instance, it would be reasonable to expect that a technical secretariat of an established verification organization would be required to stay abreast of appropriate technology for verification purposes.

ACKNOWLEDGEMENTS

The Canadian Government wishes to acknowledge the work performed under contract by the Institut Armand-Frappier. It would also be appropriate to mention that this work benefitted from support by oficials and scientists from Canada's National Research Council, Agriculture Canada and Department of External Affairs.

INTRODUCTION

A number of allegations have been made about the hostile use of trichothecene mycotoxins, in violation of the 1925 Geneva Protocol and of the 1972 Biological and Toxin Weapons Convention (1 to 3). This report in no way attempts to address those allegations.

Nevertheless, the allegations raised the question as to how one might determine if a human being had been exposed to such toxins. The Canadian Government, through the Verification Research Program of the Department of External Affairs, has examined some of the general technical, procedural and organizational problems related to the verification of allegations of the use of chemical or biological weapons. A handbook addressing some of those problems was presented to the United Nations Secretary-General on December 4, 1985. The same concerns which led to that study and report also stimulated the Canadian Government to initiate a study with a view to seeing if there might be a simple answer to the question about determining exposure of humans to trichothecene mycotoxins.

One approach to the problem might be (and has been) to collect body-fluid samples from people who might have been exposed to mycotoxins and then to package and transport the samples for analysis in sophisticated laboratories elsewhere, possibly in other countries (1 to 3). Clearly, this approach involves a considerable amount of work and expense, as well as time delays, all proceeding on a rather speculative basis. Another

approach would be to transport sophisticated, sensitive and expensive equipment to the immediate vicinity of sample collection so that the analysis might be carried out in the field. A compromise between these two approaches could involve the development of the means for a first screening in the field such that fewer samples need be sent to the laboratories for more precise analysis and confirmation.

Institut Armand-Frappier, in its initial and conceptual proposal, envisaged the development of a highly specific screening assay based on monoclonal antibodies (MAs) to test for T-2, HT-2, DAS and DON trichothecene mycotoxins in the blood. The objective was to produce a diagnostic kit which would combine the advantages of prepacking and easy transportability with those of high specificity and reliability. New test procedures involving the use of MAs - for example, in the detection of breast cancer - were considered to have a potential role to play in such a diagnostic kit (4,5). While familiar with technologies related to the development of MAs against macromolecular structures (e.g., proteins, carbohydrates, cell walls, etc.), scientists at Institut Armand-Frappier were much less familiar with the preparation of antigenic conjugates from small non-immunogenic molecules and the raising of MAs to these types of conjugates. Furthermore, these scientists were not familiar with the problems related to the manipulation of these highly toxic mycotoxins. Government scientists from Agriculture Canada and from the National Research Council of Canada, whose work was related to problems of natural contamination of foodstuffs with mycotoxins and to the preparation of hapten-conjugates respectively, provided initial assistance and advice in dealing with these problems.

This report provides a detailed record of the work performed and problems encountered by Institut Armand-Frappier, which resulted in a change in scope of the project and led to the development of a diagnostic kit based on the use of highly purified anti-T-2 polyclonal antibodies and a CELISA technique for the detection, identification and quantification of T-2 trichothecene mycotoxin in human blood. The methods and results are frankly discussed, and a number of conclusions and recommendations have been included for consideration in the event further work might be contemplated. Two "demonstrator" kits were also provided in keeping with the terms of the contract.

WORK PLAN

- September 1984 to March 1985:

  Bulk Production of Trichothecenes. This part of the study was performed at Agriculture Canada and included the following steps:

  (a) Toxin preparation,

  (b) Toxin derivatization,

  (c) Toxin purification,

  (d) Toxin assay.

- March 1985 to July 1985:

  This part of the study was also performed at Agriculture Canada and NRC and included Toxin-carrier and Toxin-enzyme preparation. The following substances were provided:

  (a) Hapten-Hemisuccinate component (T-2-HS),

  (b) Hapten-Conjugate preparation (T-2-HS-BSA),

  (c) Hapten-Conjugate analysis,

  (d) T-2 peroxidase preparation.

- July 1985 to October 1985:

  Work during this period involved the production of polyclonal antibodies and attempts to produce MAs, which included the following steps:

  (a) First immunizations using initial preparations (did not produce any detectable antibodies),

(b) Further immunization of 6 rabbits (polyclonal), and 8 BALB/c mice (monoclonal),

(c) Collection of sera at days 0, 14, 21 and 35 post immunization and sacrifice animals at day 64,

(d) Analysis of antisera produced by ELISA and/or RIA techniques,

(e) Purification of anti-T-2 antisera,

(f) Development of a CELISA technique,

(g) Fusion of spleens of immunized animals to produce MAs,

(h) Screening hybrids for the presence of anti-T-2 antibody.

- November 1985 to March 1986:

(a) Development of the CELISA screening test,

(b) Study toxicity of T-2 on different cell lines,

(c) Immunization of BALB/c mice using IgD stimulation.

- March 1986 to October 1986:

(a) Development of the "demonstrator" kit using CELISA,

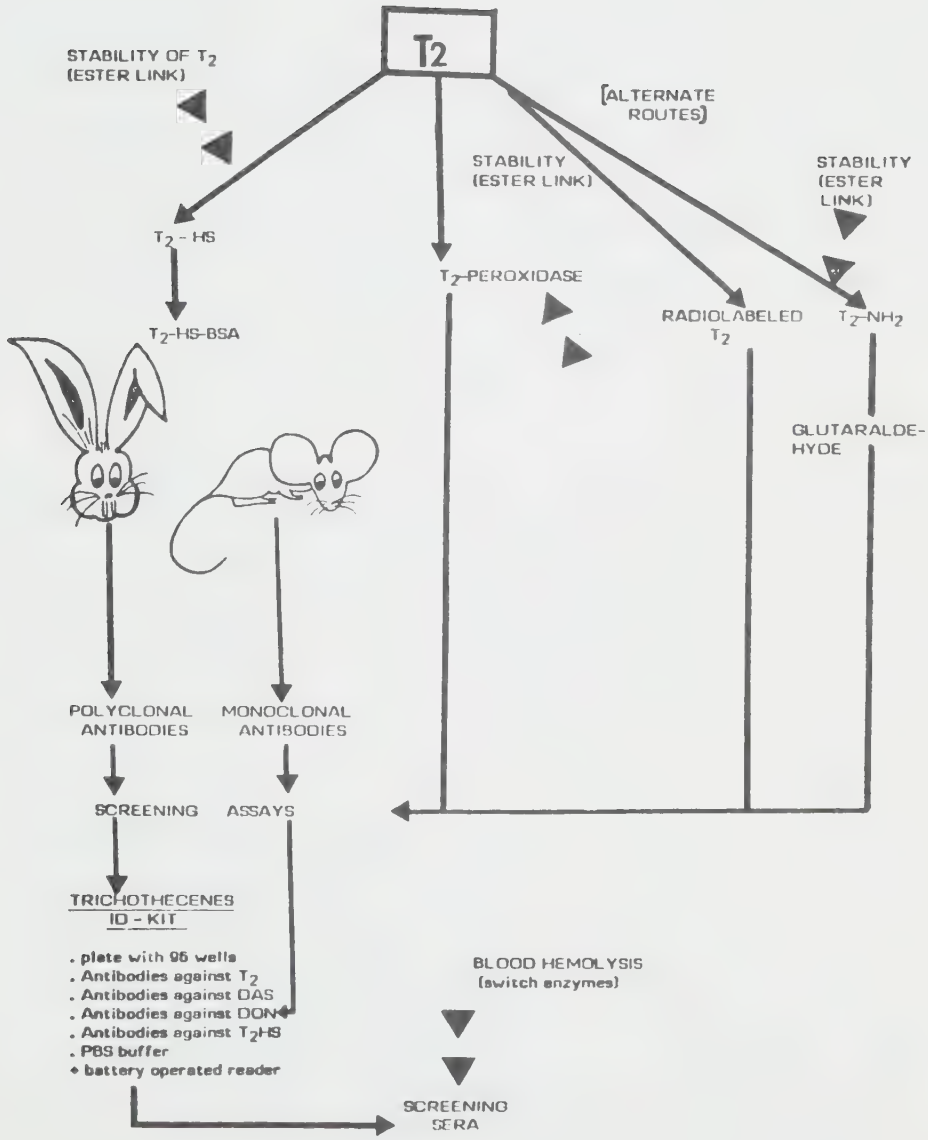
(b) Sensitivity, stability and reproducibility studies.

- October 1986 to December 1986:

Preparation of the final report.

TABLE 1

GENERAL OVERVIEW OF THE STUDY



PROBLEM AREAS

1. Hapten-Conjugate preparation: Instability of early preparations proved to be a major problem. This was probably due to the instability of the complex T-2-Hemisuccinate in the initial preparations (see Table 1).

2. Screening assay: In the process of developing the screening assay, positive sera was required but all animals immunized in the first part of the project using initial preparations showed no antibody production. This was probably due to the instability of the molecule of T-2-Hemisuccinate and the resulting low incorporation of T-2 into the BSA carrier (Table 1).

3. Production of MAs: Once the above problems were resolved, another major problem developed in the production of monoclonal antibodies. Eight different fusions were performed with the production of a limited number of hybridomas. However, these hybridomas did not produce any substantial amounts of MAs. Most of the clones developed ceased to multiply and died within 2 to 3 weeks (see Appendix B). One of the causes could have been related to T-2 toxicity; and although direct T-2 toxicity was extensively checked and proved negative, membrane transport problems could have prevented us from observing the substantial toxicity encountered.

MATERIAL AND METHODS

I. EQUIPMENT USED AT I.A.F.

1. LAMINAR FLOW HOOD:
   In order to carry out this study, a "laminar flow hood" (Bio-Guard) was purchased (IAF funds). This hood was especially equipped with a separate ventilation system so as to manipulate highly toxic mycotoxins.

2. FREEZER:
   All sera produced in this study were kept at -70°C throughout the duration of the work performed, using a Revco ultra low temperature freezer, Model 13-990-5.

3. CENTRIFUGE:
   IEC, Model CRU-5000.

4. SPECTROPHOTOMETER:
   Beckman, Model DB-GT.

5. ELISA READER:
   Mandel Scientific (Bio-Tek Instruments) EL309. Autoreader (Epson Spectrum LX DO).

6. MIXER:
   Fisher Dyna-Mix (Fisher Instruments).

7. LIQUID SCINTILLATION COUNTER:
   Beckman, Serial No. DPM-100

II. REAGENTS AND SUPPLIES:

Acetonitrile (Fisher Scientific) Catalogue No. A21-1

Amicon Membrane (Amicon Canada) 30 000 MW Catalogue No. PM 30

Ammonium Sulfate (Baker Analyzed Reagents) Catalogue No. B329

BCG: Bacillus Calmette Guerin (IAF) Lot No. 2038-2.

BSA: Bovine Serum Albumin (Sigma Chemical Co.) Fraction V Catalogue No. A-7030

Carnation Skimmed Milk

Chloroform (Fisher Scientific) Catalogue No. C-298B

CNBr: Cyanogen Bromide (Pharmacia Fine Chemicals) Catalogue No. 17-0430-01

DMF: Dimethylaminopyridine (Pierce) Catalogue No. 20672

EDC: 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide HCl (Aldrich) Catalogue No. 16, 146-2

ETOH: Ethanol, 95% (IAF)

Freund's Adjuvant: (Difco Lab. Inc.) Incomplete (FIA), Catalogue No. 0639-59; Complete (FCA) Catalogue No. 0638-59.

Glutaraldehyde Grade 1 (Sigma Chemical Co.) specially purified Catalogue No. G-6257

Glycine (Baker Analyzed Reagents) Catalogue No. M799

Goat anti-mouse IgG - POD Conjugated (Boehringer Mannheim Biochemicals) Lot No. 25021.

HCl: Hydrochloric acid, 1N (Fisher Scientific) Catalogue No. SA48-500

HFBI: Heptafluorobutyryl Imidazole (Pierce)

Microtiter Plates (Falcon) polysterene plate Catalogue No. 3040

Peroxidase Substrate Soln A/ABTS (Kikegaard and Perry Lab. Inc.)

Pertussis Vaccine: Bordetella pertussis (IAF) Lot No. 2233-4.

PVC-gloves Ambi Polyvinyl Chloride (Fisher Scientific) Catalogue No. 11-304-120C

PVP-40: Polyvinyl-pyrolidone (Sigma Chemical Co.) Pharmaceutical Grade A Mol Wt 40,000 Catalogue No. PVP-40.

SDS: Sodium Dodecyl Sulfate (Bio-Rad) electrophoresis purity Catalogue No. 161-0301

Sepharose-4B (Pharmacia Fine Chemicals) Catalogue No. 17-0430-01.

Sodium Azide (Baker Analysed Reagent) Catalogue No VO15

Sodium Bicarbonate (Baker Analysed Reagent) Catalogue No. 3506.

Sodium Carbonate (Baker Analysed Reagent) Catalogue No. 4502

Sodium Chloride (Baker Analysed Reagent) Catalogue No. 3624

Sodium Citrate (Baker Analysed Reagent) Catalogue No. 99507-3

Sodium Phosphate (Baker Analysed Reagent) Catalogue No. 3818

Succinic Anhydride (Bio-Pharma) Catalogue No. 57626

T-2-BSA Conjugate (Sigma Chemical Co.) Catalogue No. T-9642

T-2 Toxin (Sigma Chemical Co.) Catalogue No. T-4877.

^{3}H-T-2-Toxin (Amersham Corp.) B-(3-^{3}H) T-2-Toxin, specific activity 11 Ci/mmol Product Code TRQ. 4033

THF: Tetrahydrofuran (Bio-Pharma) Catalogue No. T5267

Toluene (Mallinckrodt) Catalogue No. 8608

Trichothecene Kit (Sigma Chemical Co.) Catalogue No. TK-8 contains T-2, HT-2, T-2-triol and T-2-tetraol

Tris: Tris (Hydroxymethyl) aminomethane (Bio-Rad) Catalogue No. 161-0716

Tween-20 (Sigma Chemical Co.) Catalogue No. P-1379

Whatman No 1 filter paper (Canlab) Catalogue No. 09-805A

III. BUFFERS AND SOLUTIONS

a. Phosphate Buffer Solution (PBS)

0.9 g of NaH_2PO_4

3.6 g of Na_2HPO_4

17 g of NaCl

1 900 ml of distilled water

Adjust pH to 7.5 with HCl

Make the volume to 2 000 ml with distilled water

Filter solution through a Whatman no. 1 filter paper

b. Ammonium Sulfate Preparations:

(i) Saturated Ammonium Sulfate

50 g of ammonium sulfate

50 ml of distilled water

Heat solution to 50°C, adjust pH to 7.5 with NaOH 1N

Filter solution through a Whatman no. 1 filter paper

(ii) 50% Ammonium Sulfate

50 ml of saturated ammonium sulfate

50 ml of Phosphate Buffer mentioned in (a)

Adjust pH to 7.5 with NaOH 1N

Filter solution through a Whatman no. 1 filter paper

(iii) 33% Ammonium Sulfate

33 ml of saturated ammonium sulfate

67 ml of Phosphate Buffer mentioned in (a)

Adjust pH to 7.5 with NaOH 1N

Filter solution through a Whatman no. 1 filter paper

c. Sodium Dodecyl Sulfate (5%) (ELISA Stopping Reagent)

5 g of SDS

100 ml of distilled water

d. Blocking Buffer

(i) Citrated Milk: 100 g of Carnation Skimmed Milk

44.1 g sodium citrate

Complete volume to 500 ml with distilled water

Dialyze against distilled water for 48 hours at 4°C.

(ii) TBS Buffer: 4.84 g of Tris

54.48 g of NaCl

Add 1 900 ml of distilled water

Adjust pH to 7.5 with HCl.

Make the volume to 2 000 ml with distilled water

Filter solution through a Whatman no. 1 filter paper

(iii) Working Solution: 20 ml of citrated milk

1 g of PVP-40

5 g of BSA

Add 75 ml of TBS Buffer

Adjust pH to 7.5 with NaOH 1N

Make the volume to 100 ml with distilled water

Filter solution through a Whatman no. 1 filter paper

e. Washing Buffer

TBS Buffer (as mentioned in d. ii)

0.1% Bovine Serum Albumin

0.05% Tween-20

f. Dilution Buffer

0.5 g of PVP-40

5 g of BSA

90 ml of TBS Buffer

Adjust pH to 7.5 with NaOH 1N

Make the volume to 100 ml with distilled water

Filter solution through a Whatman no. 1 filter.

g. Glycine Buffer 0.1 M pH 2.8 and pH 11.0

0.75 g of glycine

Add 90 ml of distilled water

Adjust pH with HCl 1N for pH 2.8

Adjust pH with NaOH 1N for pH 11.0

Make the volume to 100 ml with distilled water

Filter solution through a Whatman no. 1 filter

h. Citrate Coupling Buffer

58.8 g sodium citrate 0.2M

29.8 g sodium chloride 0.5M

Dilute with 1 liter of distilled water

Adjust pH to 6.7 with HCl.

i. BSA-Sepharose-4B Affinity Column

(i) CNBr-Activated Sepharose-4B

In a fume hood, 30 ml of packed Sepharose-4B was washed with 200 ml of distilled water and resuspended in 60 ml of distilled water in a beaker. After mixing (for 1 hr or until the preparation is well mixed) 1 g of cyanogen bromide per 10 ml of gel (3 g in 1-2 ml of acetonitrile, 99% for

30 ml of gel) was added. The pH of the preparation was maintained between 10 and 11 with NaOH 1M, for 15 min. The preparation was then transfered to a funnel of medium porosity and successively washed with 10 volumes of cold distilled water and 10 volumes of cold citrate coupling buffer (Fig. 1).

(ii) Coupling of BSA to CNBr-Activated-Sepharose-4B

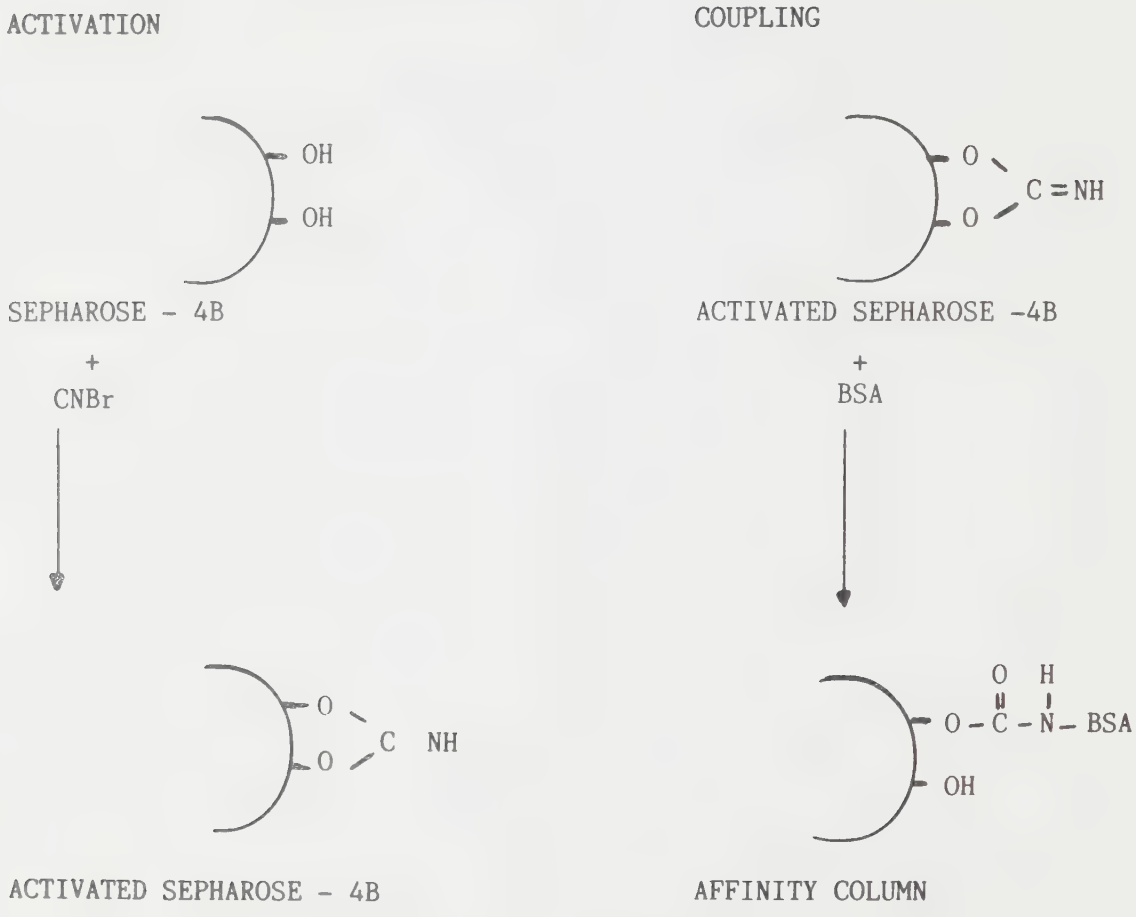
The CNBr-activated Sepharose-4B was added to 60 mg of BSA disolved in 30 ml of citrate coupling buffer. Coupling was performed under refrigeration condition (4°C) for 20 hrs. An equal volume of 0.1M ethanolamine was added to the gel and left for 2 hrs at 4°C. This was followed by extensive washing with 10 volumes of sodium acetate 0.1 M, NaCl 0.4 M pH 4.0, 10 volumes of sodium bicarbonate 0.1 M, NaCl 0.5 M (pH 10) and finally with 10 volumes of carbonate buffer 0.01 M, NaCl 0.15 M (pH 9.5). The column was kept at 4°C in presence of Na azide 0.02% (Fig. 1).

(iii) Purification of Antibody Preparations

The BSA column was first washed with glycine buffer pH 2.8 then with glycine buffer pH 11.0, and finally with PBS pH 7.0. 10 ml of rabbit anti-T-2-antisera was then added to the column. This was followed by elution with PBS pH 7.0. Fractions (5 ml) were collected and read by a spectrophotometer at an absorbance of 280 nm = A_{280}. The fractions containing the antibody were pooled. The column was then washed with glycine buffer pH 2.8 and pH 11 and finally with PBS containing 0.1% sodium azide.

FIGURE 1

PREPARATION OF AFFINITY COLUMN



IV. TECHNIQUES

1. Hapten-Hemisuccinate Preparation

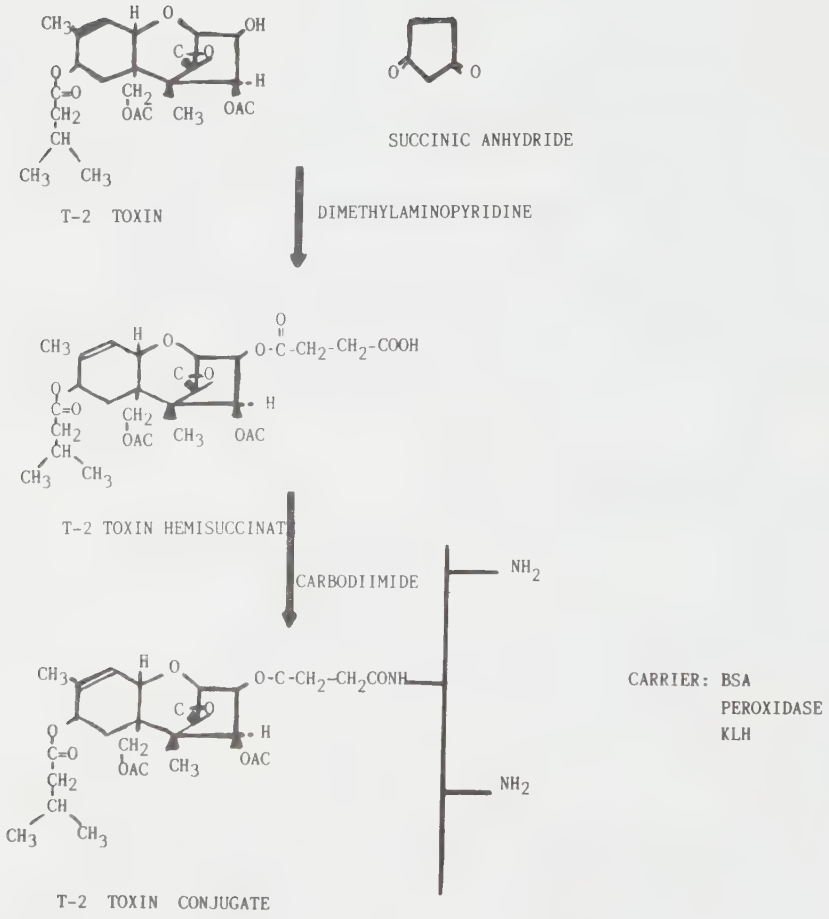
T-2 (5 mg) was dissolved in a mixture containing THF (1 ml) and dimethylaminopyridine (5 mg). Succinic anhydride (purified mp 119°C) (100 mg) was added and stirring continued overnight at room temperature (7), then 10 ml of chloroform was added and the chloroform layer washed three times with water (10 ml) then evaporated to dryness. The residue was dissolved in DMF (1 ml) and an aliquot added to the conjugate preparation (Fig. 2).

2. Hapten-Conjugate Preparation

BSA (12.5 mg) was dissolved in distilled H_2O (12.5 ml) and EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl) (7.5 mg) was then added. This was followed by the addition of the solution of T-2 hemisuccinate in DMF (1 ml) over 5-10 min. A further amount of EDPC (5 mg) was added and the stirring continued at room temperature for 16 to 24 hours. The solution was then dialyzed at 4°C against 3 changes of distilled H_2O. Protein (Lowry) and T-2 concentrations were determined. When necessary, the solution was concentrated on a Minicon A5 filter to 1 to 1.5 mg/ml and the protein and T-2 concentration reassessed.

FIGURE 2

HAPTEN-CONJUGATE SYNTHESIS



3. Hapten-Conjugate Analysis for Mycotoxin

1 N NaOH (0.1 ml) was added to the sample (1 ml) and hydrolysis was allowed to proceed at room temperature for 30 min. An aliquot (100 ul) was mixed with MeOH (2 ml) and evaporated to dryness under N_2. Heptafluorobutyrylimidazole (HFBI) (100 ul) and toluene/acetonitrile 95:5 (1 ml) were then added. The mixture was heated in a sealed vial at 80°C for 60 min then allowed to cool to room temperature. 5% w/v Na_2CO_3 (1 ml) was added and the preparation and vortexed to mix. An aliquot (25 ul) of the organic phase was analyzed by capillary GC (Varian, 30 m x 0.5 mm, DB5, electron capture detection).

4. Toxin-Peroxidase Preparation

T-2 (1.5 mg), dimethylaminopyridine (1.5 mg) and succinic anhydride (30 mg) were mixed in THF (0.3 ml, purified and stored under N_2) and stirred overnight at room temperature. Chloroform (10 ml) was added and the lower organic phase washed with water (1 volume 4x). The chloroform was evaporated to dryness under N_2. The residue from the 1.5 mg T-2 hemisuccinate preparation was dissolved in EtOH (2 ml) and H_2O (6 ml) was added. This solution was immediately added to a freshly prepared mixture of EDC (300 mg) and peroxidase (6 mg) in 25% v/v ethanol/water (2 ml). The resulting mixture was stirred at room temperature (30 min) and a further amount (300 mg) of EDC added. Stirring was continued overnight at 4°C. The solution was dialyzed (Spectropor 3, 3500 MW cut-off) against 3

changes (24 hrs each) of 0.01 M sodium phosphate pH 7.5. The dialyzate was analyzed for protein concentration (Lowry) and adjusted to contain (0.2 to 0.3 mg peroxidase per ml). Mycotoxin analysis was also performed. Aliquots (30-50 ul) were then frozen away for storage.

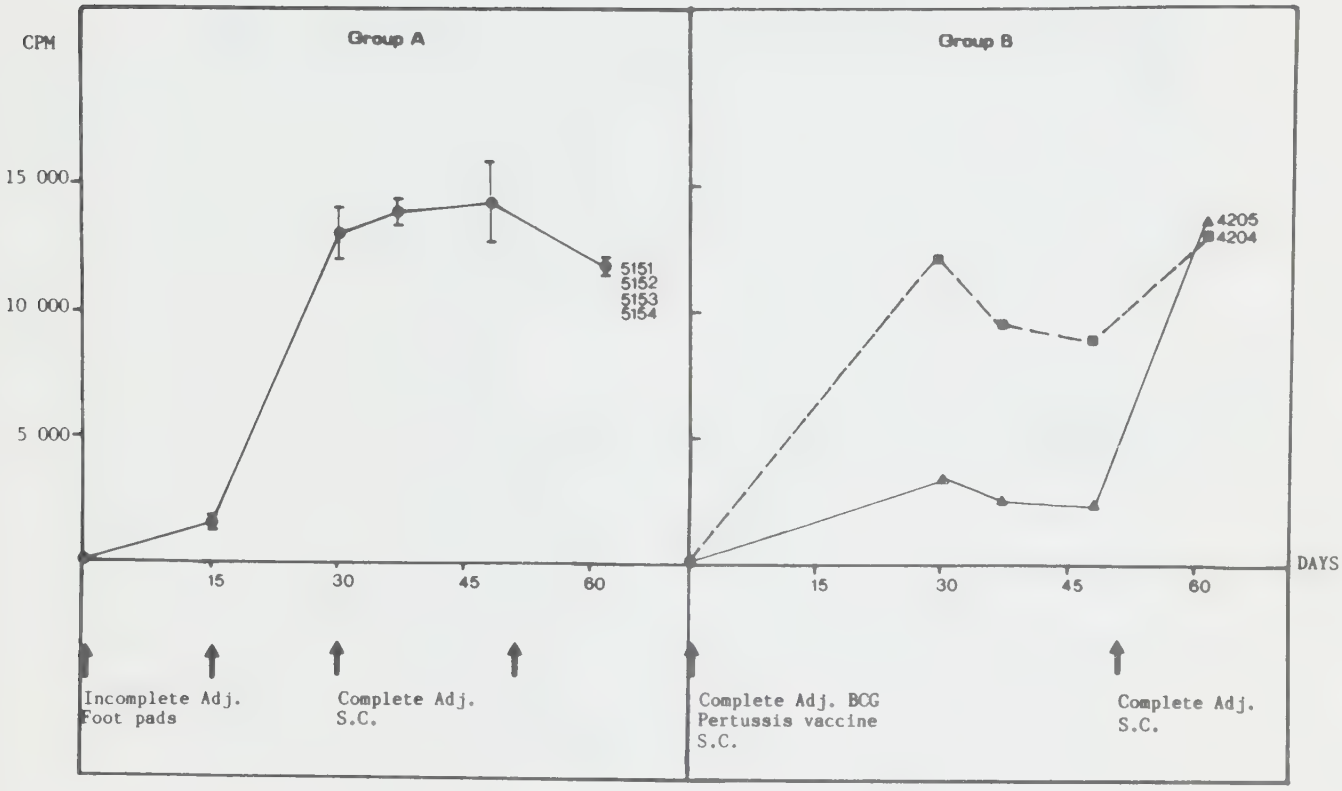
5. Polyclonal Antibody Preparation:

Six female New Zealand rabbits (3-3.5 kgs) were used for the production of anti-T-2 antibodies (6). Control sera (5 ml) were collected from each of the animals before immunization by bleeding from the ear lobe. Plasma was separated from the buffy coat and cells, then frozen for future testing. These animals were divided into 2 groups (Fig. 3):

GROUP A: Four animals (5151, 5152, 5153, 5154) were injected with the contents of one vial containing Hapten-Conjugate (1 ml) (protein concentration approximately 1 mg/ml), mixed with an equal volume of Freund's incomplete adjuvant (FIA). Each rabbit received 100 ul under each of the two front foot pads, i.e. 200 ul total/rabbit. After two weeks, an identical mixture was injected under the two rear foot pads (200 ul total). The foot pad route was selected to minimize the animal's exposure to toxin. Two weeks later, a mixture of Hapten-Conjugate (1 ml) containing an equal volume of Freund's complete adjuvant (FCA) was injected subcutaneously (s.c.) in the dorsal region (intrascapular zone); six sites received injections (100 ul). After one week, sera (5 ml) were collected from each rabbit and frozen for testing later.

FIGURE 3

IMMUNIZATION PROTOCOLS AND TITRATION OF ANTI-T-2 ANTISERA



Two weeks after the s.c. injection, a booster dose was given with Hapten-Conjugate (1 ml) mixed with an equal volume of FCA (dorsal s.c. injections 6 x 100 ul) (Fig. 3).

GROUP B: Two rabbits (4204, 4205) were injected with 1 ml of a preparation containing Hapten-Conjugate (1 ml) and BCG (50 mg) mixed with 1 ml of FCA at multiple sites in the back together with 0.5 ml of Pertussis vaccine. After fifty days these animals received a booster dose of 1 ml of Hapten-Conjugate (500 ug) in FCA at multiple sites in the back (Fig. 3).

6. Monitoring of Anti-T-2 Antibody Production:

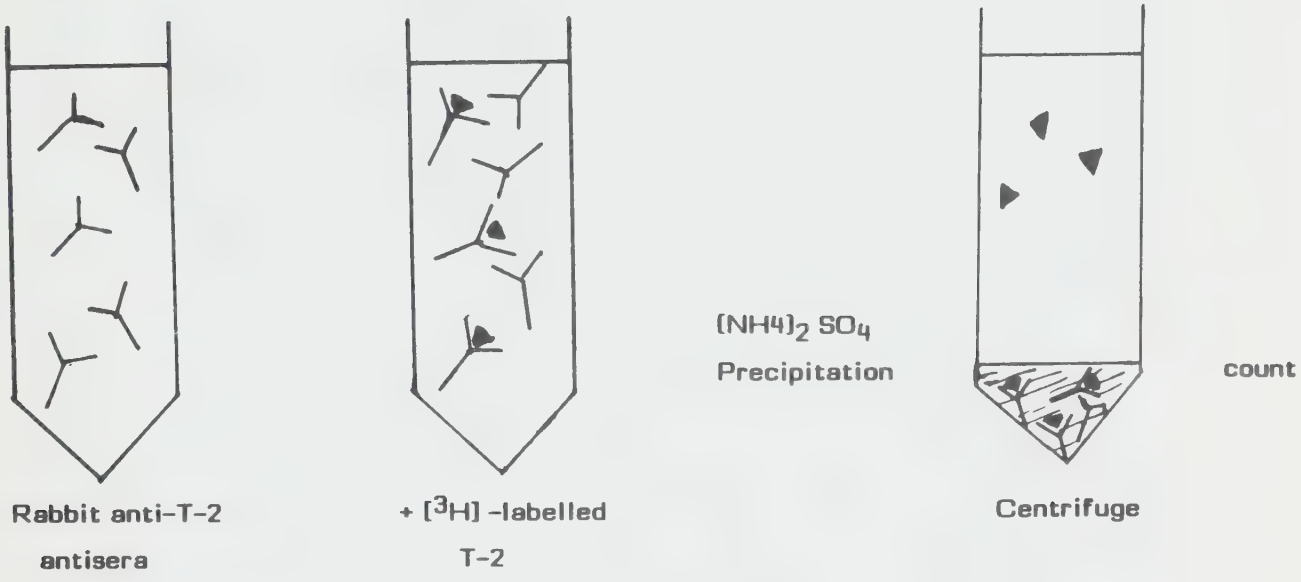
Animals in both groups were bled (from the ear lobes) at regular intervals for antibody titration. All animals were sacrificed at day 64 by intracardiac bleeding. Monitoring of anti-T-2 antibody production was performed using either RIA or ELISA techniques.

a. Radioimmunoassay (RIA):

Rabbit sera were diluted serially in PBS (two-fold dilution going from 1/2 to 1/128). Then in a small conical plastic tube, PBS (100 ul) was added to the serially diluted rabbit serum (20 ul). To this solution was added 30 ul of (^{3}H)-labelled T-2 Toxin (specific activity, 11Ci/mM) containing approximately 42 000 cpm. The mixture was then incubated for 3 hrs at room temperature (20°C to 30°C). After this incubation, an ammonium sulfate precipitation (50%) was performed (Fig. 4). The precipitate was collected by centrifugation (8 000 rpm for 10 min at room temperature) and the precipitated radioactivity counted in scintillation vials.

FIGURE 4

RIA TECHNIQUE FOR SCREENING ANTIBODY PRODUCTION



7. Purification of Anti-T-2 Antibodies

Purification of antibodies was done in two steps. First by ammonium sulfate precipitation then by BSA affinity column purification.

a. Ammonium Sulfate Precipitation

Rabbit immune sera were partially purified at room temperature by three (55%, 33% and 33%) sequential ammonium sulfate precipitations as previously described (4,5). For this step ammonium sulfate was added drop by drop to the preparation of immune sera under constant agitation. The preparation was then transferred into a 50 ml conical tube and placed at 4°C for 16 hrs. The precipitate was recovered by centrifugation at 8 000 rpm for 20 min at 4°C, dissolved in PBS pH 7.3 and dialyzed for 16 hrs against the same buffer.

b. Affinity Column Purification

Because T-2 BSA complex was used as immunogen and in order to adsorb anti-BSA antibodies, we used a BSA-Sepharose 4B column prepared according to the technique already described on page 13 and 14.

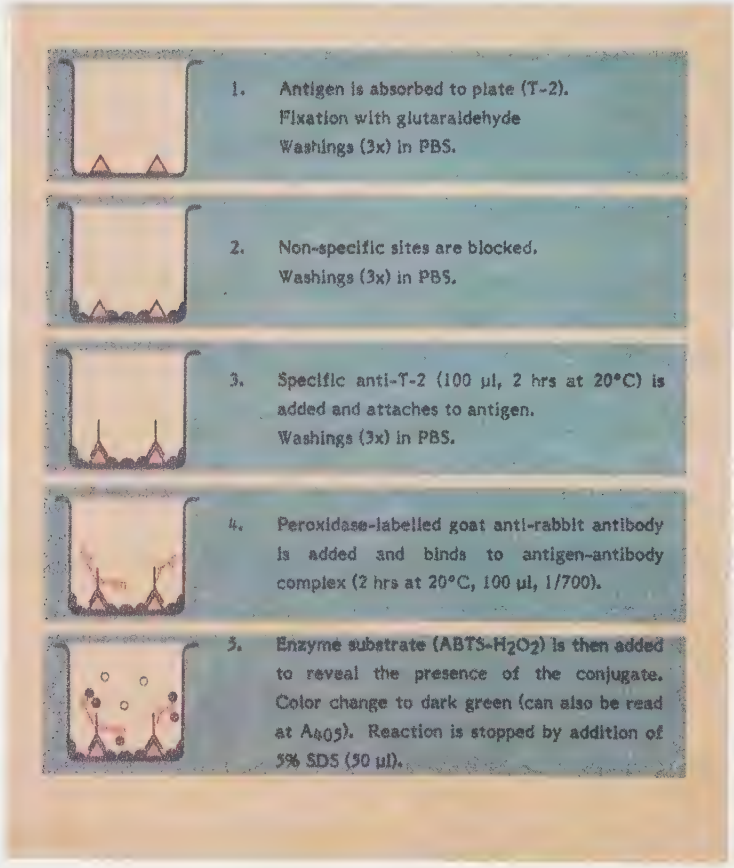
8. Enzyme-linked ImmunoAssay (ELISA):

In the ELISA procedure, a standard technique was performed essentially as described previously (4,5). Polystyrene microtitre plates were used as the solid phase. They were coated with 0.2 ml of a preparation of T-2-BSA (100 ug/ml in PBS) and incubated overnight at 4°C. The plates were emptied over a filter paper and the wells fixed with 0.2 ml of 0.2% glutaraldehyde solution for 1 hr at room temperature. After 3 washings in Washing Buffer, the non-specific sites were blocked by the Blocking Buffer. Plates were then extensively washed with Washing Buffer and either immediately used or stored on shelves at room temperature (Fig. 5).

For antibody titration, anti-T-2 serum were serially diluted 1/10 in Dilution Buffer and 100 ul were added to each well of the plate. After a 2 hr incubation at 20°C and 3 washings in Washing Buffer, 100 ul of the enzyme (horseradish peroxidase)-labelled goat anti-rabbit conjugate (diluted 1/700 with Dilution Buffer) was added to each well and the plate was incubated for 2 hrs at 20°C. The reaction was revealed by 100 ul of ABTS-hydrogen peroxide substrate. The reaction was stopped by the addition of 50 ul of 5% Sodium Dodecyl Sulfate (SDS) solution and the total bound enzyme activity was determined spectrophotometrically at A_{405}. In order to decrease nonspecific binding, the Washing Buffer contained 0.1% BSA.

FIGURE 5

STANDARD ELISA FOR SCREENING ANTIBODY PRODUCTION

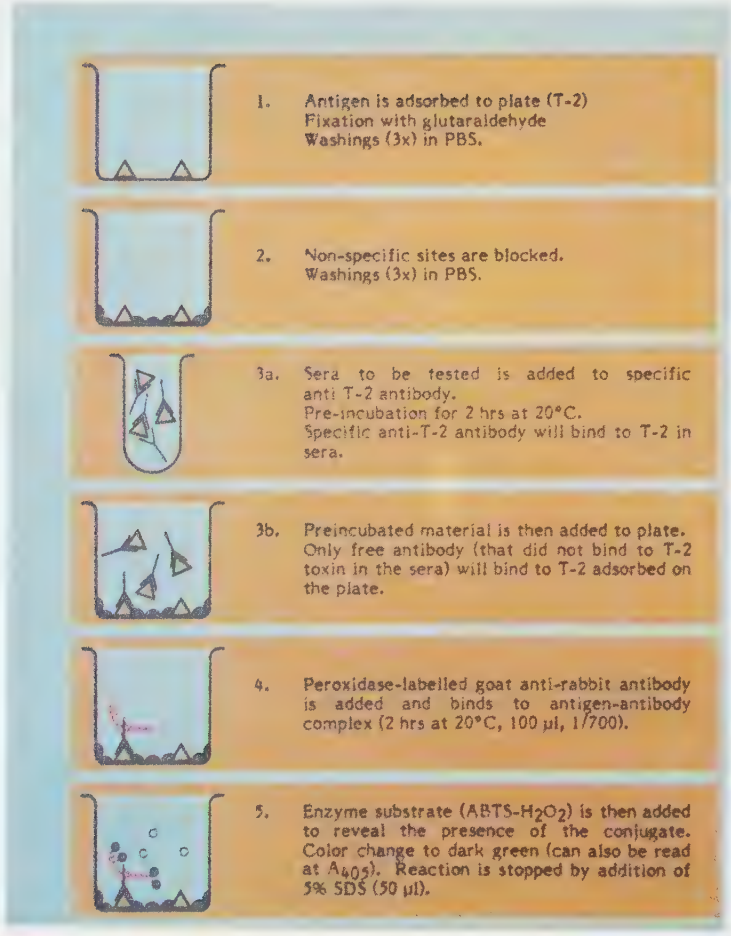


9. Competitive ImmunoAssay (CELISA)

For competitive ELISA (Fig. 6), 100 ul of standard T-2 toxin (at different dilutions) was added to 100 ul of anti-T-2 serum (1/10 000) and incubated overnight at 4°C or for 2 hrs at 20°C before its use in the ELISA assay as described above. Because the most sensitive point to be at used in competition assays is usually considered to be at the 50% reactivity point of the serum, the 1/10 000 dilution of anti-T-2 antiserum (as observed in the titration assay) was used to set up a standard curve for T-2, BSA and T-2-BSA. This dilution was determined for all the rabbit antisera developed and proved to be approximately the same for all of them (see pages 31 to 34).

FIGURE 6

CELISA FOR DETECTION OF T-2 IN THE BLOOD



RESULTS

1. PRODUCTION OF ANTI-T-2 ANTIBODY IN RABBITS

As detailed in the Material and Methods section, six female New Zealand rabbits were divided into 2 groups:

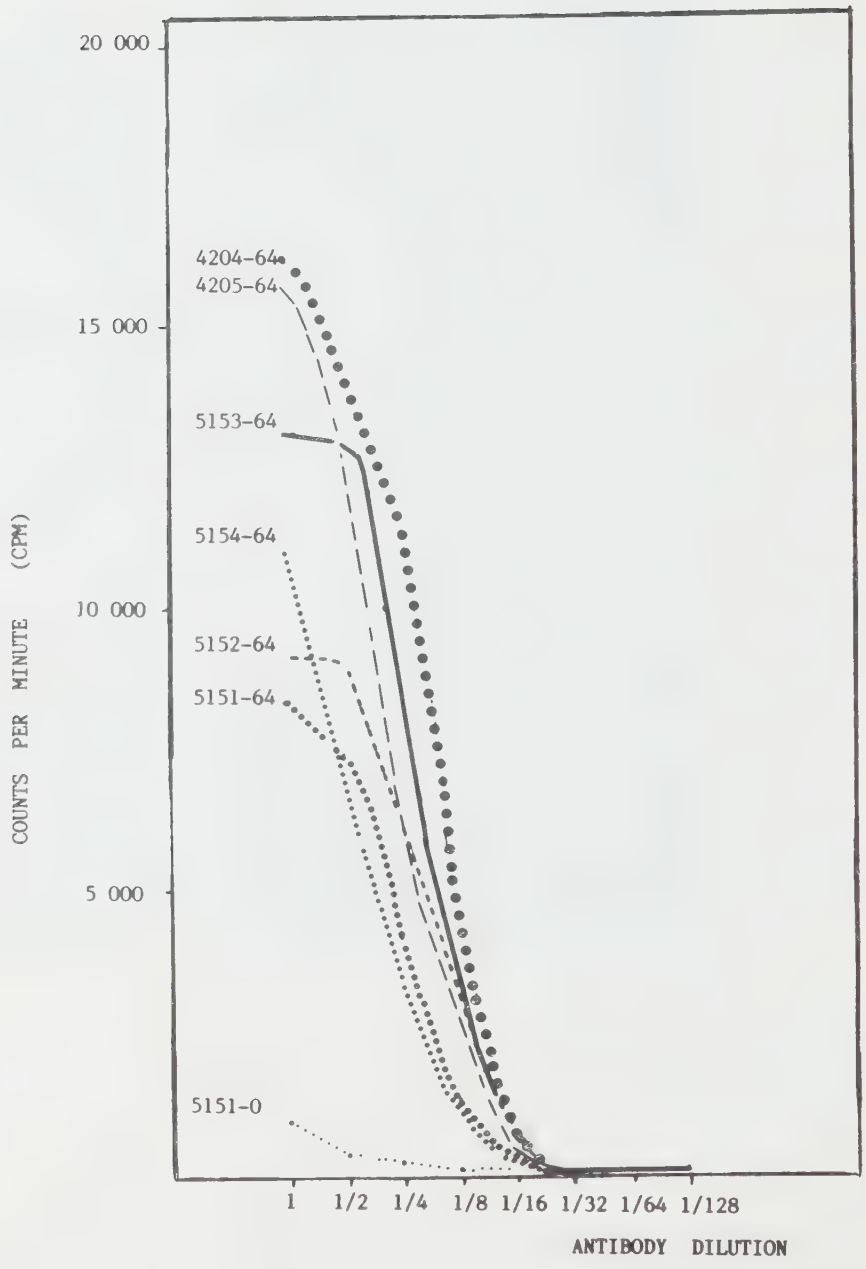
Group A - Animals no. 5151, 5152, 5153 and 5154

Group B - Animals no. 4205, 4204

These animals were bled at regular intervals (Fig. 3, page 20) and sacrificed at day 64 by intracardiac bleeding. Levels of anti-T-2 antibody production were monitored by RIA using (^{3}H)-labelled T-2. Results of antibody titration are also given in Fig. 3. This figure is a summary of antibody titration on each day showing the kinetics of antibody production, i.e. increase in anti-T-2 antibodies (as revealed by RIA) in the sera of animals after each immunization. Data on animals in group A were pooled together and given as means with standard deviation (vertical bars). Data clearly show that animals in group B (4204 and 4205) gave the best antibody response, illustrating that the best and simplest immunization protocol was that performed for group B where animals were first injected with a preparation containing Hapten-Conjugate (1 ml) and BCG (50 mg) mixed with 1 ml of FCA at multiple sites in the back together with 0.5 ml of Pertussis vaccine. This was boosted after fifty days with a preparation containing Hapten-Conjugate (500 ug) in FCA at multiple sites in the back.

FIGURE 7

RIA TITRATION CURVES



2. ANTI-T-2 ANTIBODY TITRATION

Using RIA technique, the antibody level in each of the 6 different antisera was titrated using 8 different concentrations, Fig. 7. Titration was performed only on sera collected at the day of sacrifice (D 64) and compared to sera collected before immunization (D-0). These titrations curves showed that animals in group B (4204 and 4205) gave the best antibody response.

3. ANTI-T-2 ANTIBODIES PURIFICATION

For this study, rabbit (4204) was selected at day 64 (4204-64). Immune sera was first partially purified by sequential ammonium sulfate precipitations (55%, 33%, 33%), dialysed extensively against PBS and then concentrated by ultrafiltration using an Amicon membrane (4204-64C). The preparation was then further purified on a BSA-Sepharose-4B column in order to remove the anti-BSA antibodies from the serum. Elution of the column was performed with 0.07 M sodium carbonate NaCl 0.15 M pH 2.5. Each step of the purification was monitored by both RIA and ELISA assays. Table 2 is an illustration of the results obtained using antisera from Rabbit No. 4204.

4. ELISA FOR ANTIBODY TITRATION:

Using an ELISA technique, we first titrated anti-T-2 antisera raised in animal 4204 at day 64 and compared it to serum from the same animal before immunization. Figs. 8 and 9 clearly shows that even at a 1/100 000 dilution, rabbit anti-T-2 antiserum can still give a good reaction that can be detected by this technique.

TABLE 2

MONITORING ANTI T-2-ANTIBODIES PURIFICATION

ANTISERUM	RIA (cpm)	ELISA* (A_{405})
4204-0	-	0.6
4204-64	67 000	0.8
4204-64C	145 000	1.8
4204-64A	127 000	1.4

* The same antisera (4204) was used at 1/10 000 dilution in PBS.

4204-0 Serum of rabbit no 4204 before immunization

4204-64 Native antisera before purification

4204-64C Concentrated antisera (antisera after ammonium sulfate precipitation and concentration on amicon filters)

4204-64A Adsorbed antisera

cpm Counts Per Minutes

Using 3 different dilutions (1/1 000, 1/10 000, 1/100 000), we then screened all of the anti-T-2 antisera produced in order to establish the dilution that shows 50% reactivity. (This is the dilution point that is always used in the construction of a competition assay.) Table 3 clearly shows that for all antisera, this dilution was approximately the same i.e., between 1/10 000 and 1/15 000.

FIGURE 8

ELISA TITRATION CURVES

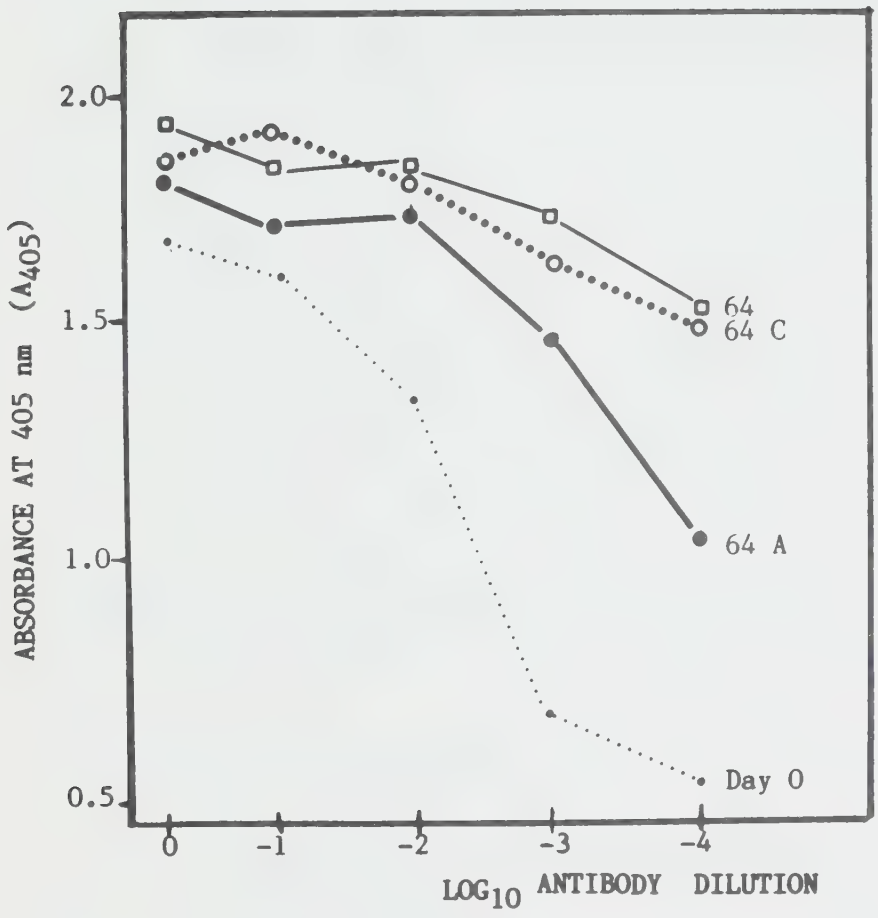


FIGURE 9

SCREENING OF ANTI-T-2 ANTIBODY AFTER EACH PURIFICATION STEP

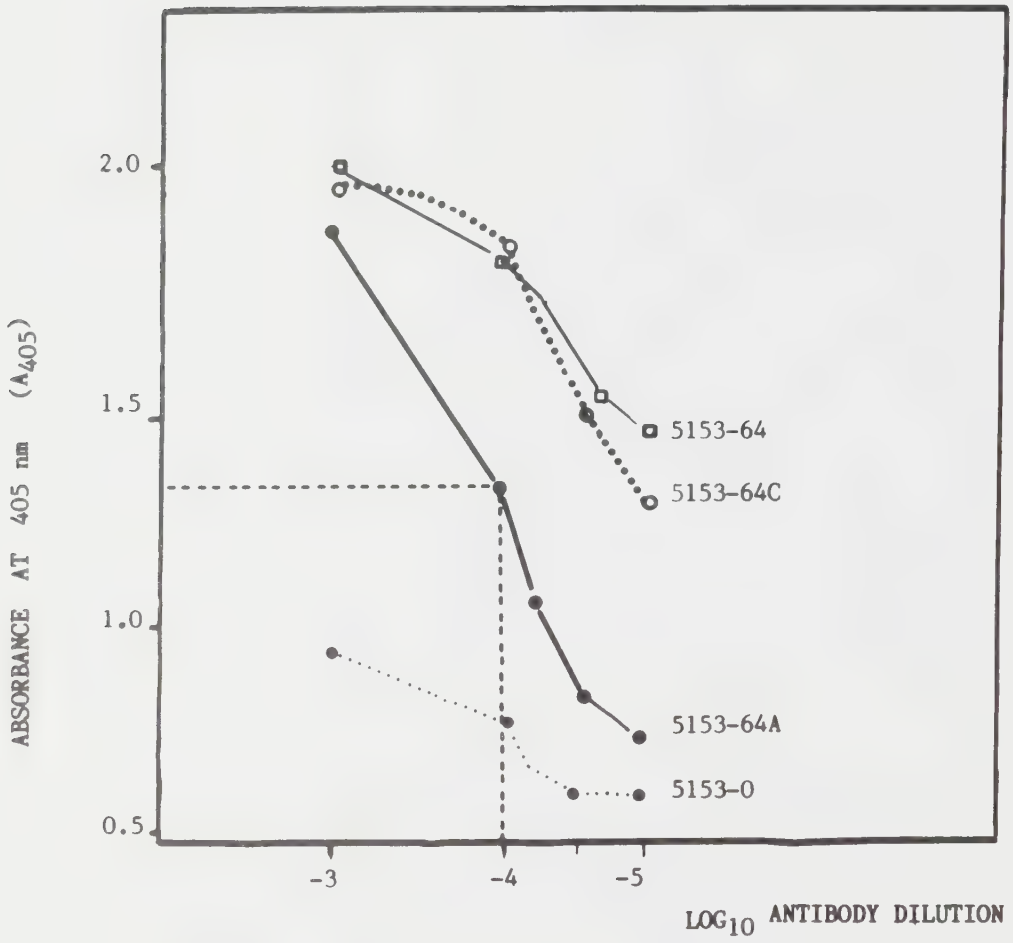


TABLE 3

ANTISERA DILUTION SHOWING 50% REACTIVITY IN ELISA

Rabbit anti-T-2 Antisera	Dilution showing 50% reactivity
4204-A	1/10 000
4205-A	1/10 000
5154-A	1/15 000
5153-64	1/10 000

4204-A, 4205-A, 5154-A = adsorbed antisera
5153-64 = antisera at day 64

5. CELISA FOR DETECTION OF T-2 IN THE BLOOD

In order to establish the sensitivity of our technique, we established a CELISA (Competitive ELISA) technique in which an additional step is added (step no 3a, see Fig. 6, page 27). In this step, the anti-T-2 antibody is preincubated with increasing amounts of T-2, BSA and T-2-BSA in order to establish the lowest concentration of T-2 that will still show a clear difference in the color (and Absorbance at A_{405}) compared to the control. Results shown in Figs. 10 and 11 clearly demonstrate that the presence of very small amounts of T-2 in the serum of an individual to be tested will induce a change in color, i.e. 50% inhibition at 10^{-7} M and 30% inhibition at 10^{-8}M.

FIGURE 10

CELISA (COMPETITIVE ELISA)

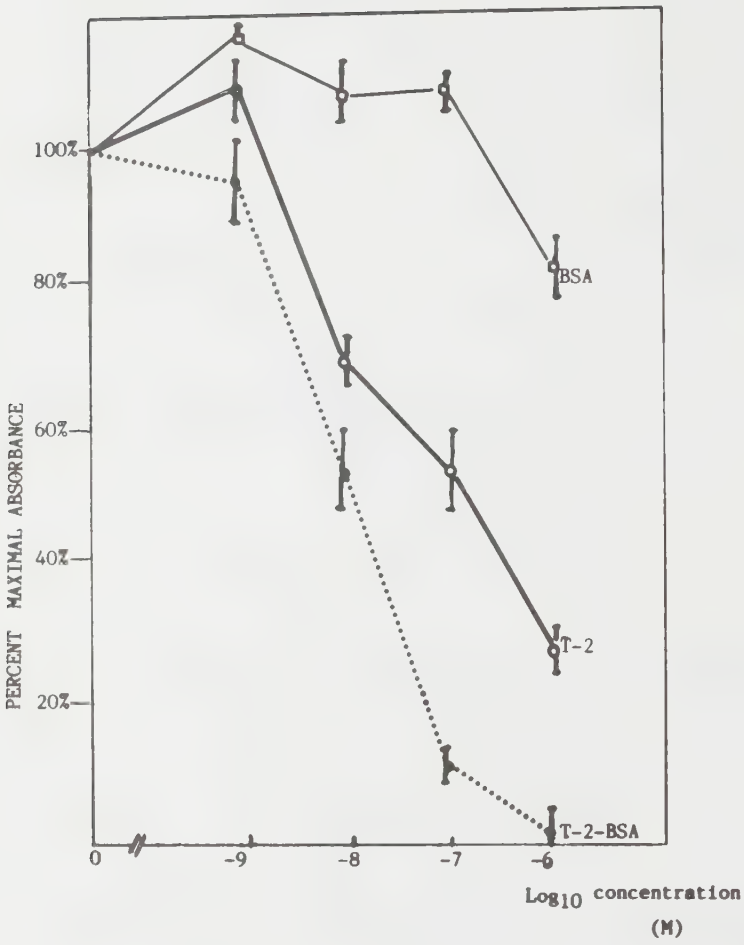
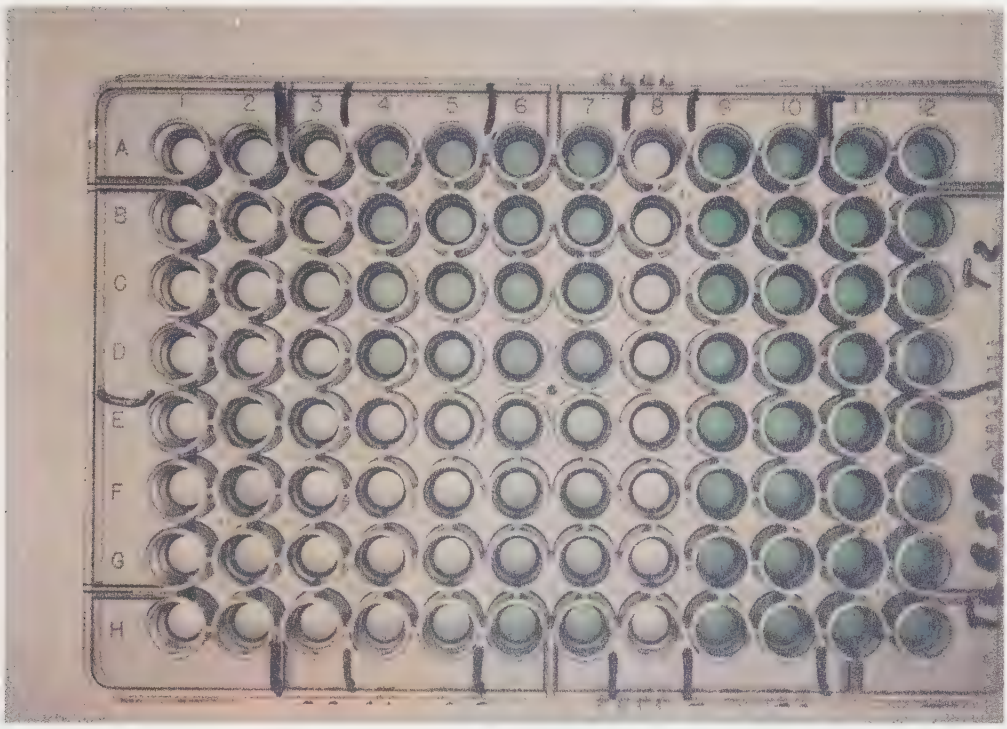


FIGURE 11

RESULTS OF CELISA TEST USING DIFFERENT CONCENTRATION OF ANTIGEN



Samples containing 3 different concentrations of added T-2 or T-2-BSA (as shown on plate) are being tested in duplicate in rows 4 to 10 (4 and 5= 0.5 ug; 6 and 7= 5 ng; 9 and 10= 0.1 ng; weight relate to T-2 in all cases).

Rows 1, 3 and 8 are empty.

Row 2 is a negative color control and represent T-2-BSA in wells without addition of anti-T-2 antisera.

Rows 11 and 12 are positive color controls prepared by binding T-2-BSA to the wells and incubating with 100 ul of anti-T-2 antisera (Fig. 6, page 27).

The graduation of color observed denotes the sensitivity of the kit and varies from very light green (Rows 4 and 5; inhibition of color at 0.5 ng of T-2 or T-2-BSA) to very dark green (Rows 9 and 10; no inhibition of color at 0.1 ng of T-2 or T-2-BSA). Rows 6 and 7 represent 5 ng of T-2 or T-2-BSA and give an in between inhibition.

Note that, when used at the same concentration, T-2-BSA seems to be a better inhibitor of the reaction that T-2. This is mainly due to the fact that T-2-BSA has been used as immunogen for the production of anti-T-2 antibodies.

DISCUSSION

The objective of this work was to produce a diagnostic test which would combine the advantages of prepacking and easy transportability with those of high specificity and reliability for the detection of T-2 toxin in the blood. A number of problems were encountered during the development of our kit:

A. Early during the development of anti-T-2 antibodies, problems associated with the instability of the Hapten-Hemisuccinate and Hapten-Conjugate preparations and the detection of anti-T-2 antibodies were encountered. During this period, the collaboration of Drs. Williams and Cohen (in attempts to develop a primary label system incorporating only peroxidase enzyme and no radiolabelled T-2) was greatly appreciated. However, we could only solve the problem by the acquisition of (^{3}H)-labelled T-2 from Amersham. This product helped us establish the presence of anti-T-2 antibody in the rabbit antisera. The problem was not, therefore, in the immunization of animals but rather in the detection of the antibodies produced (Table 1, page 6). Once the presence of anti-T-2 antibodies was proven, we proceeded to develop, the second antibody assay (ELISA) as the non-radiolabelled detection system.

B. In the development of anti-T-2 monoclonal antibodies (as detailed in Appendix B) a long time was spent in the establishment of viable hybridomas. Eight fusions were performed and, in all of these fusions, we could see viable hybridomas. These died, however, after 1 or 2 weeks. The problem could not be solved even by IgD

stimulation (8,9). Meanwhile, polyclonal antibodies were extensively purified and were shown to be highly sensitive. A further step was introduced in order to compare the sensitivity of this technique to the already-published data by Hunter et al (reference no 10) using monoclonal antibodies (Fig. 12). Using anti-T-2 polyclonal rabbit antibodies diluted at 1/10 000 in a CELISA assay, it was shown that 2.1×10^{-8} M of T-2 could induce 50% inhibition. This inhibition (as also demonstrated in Fig. 11) can easily be observed by naked-eye examination. In the case of monoclonal antibodies produced by Hunter, 1×10^{-6} M of T-2 induce the same effect (Fig. 12). We were therefore very confident of the performance of our highly purified polyclonal antibodies.

In view of these encouraging results and of certain problems encountered in the production of MAs, permission was requested to focus attention on the development of a diagnostic kit using purified and highly sensitive polyclonal antibodies.

C. The TSK/T-2 kit was developed using a CELISA technique (detailed in appendix C); and two "demonstrator" kits were provided.

D. The amounts of anti-T-2 antisera stored by IAF (at -70°C) and the approximate number of assays that could be performed using these materials are presented in Table 4. With the purified materials in hand, a total of 780 000 assays could be performed and approximately similar numbers could be expected to be run with the native and concentrated materials, after their respective purification. If required, additional materials could be produced by further immunizations of animals, now that the procedure has been developed.

TABLE 4

AMOUNT OF ANTISERA AVAILABLE FOR CELISA

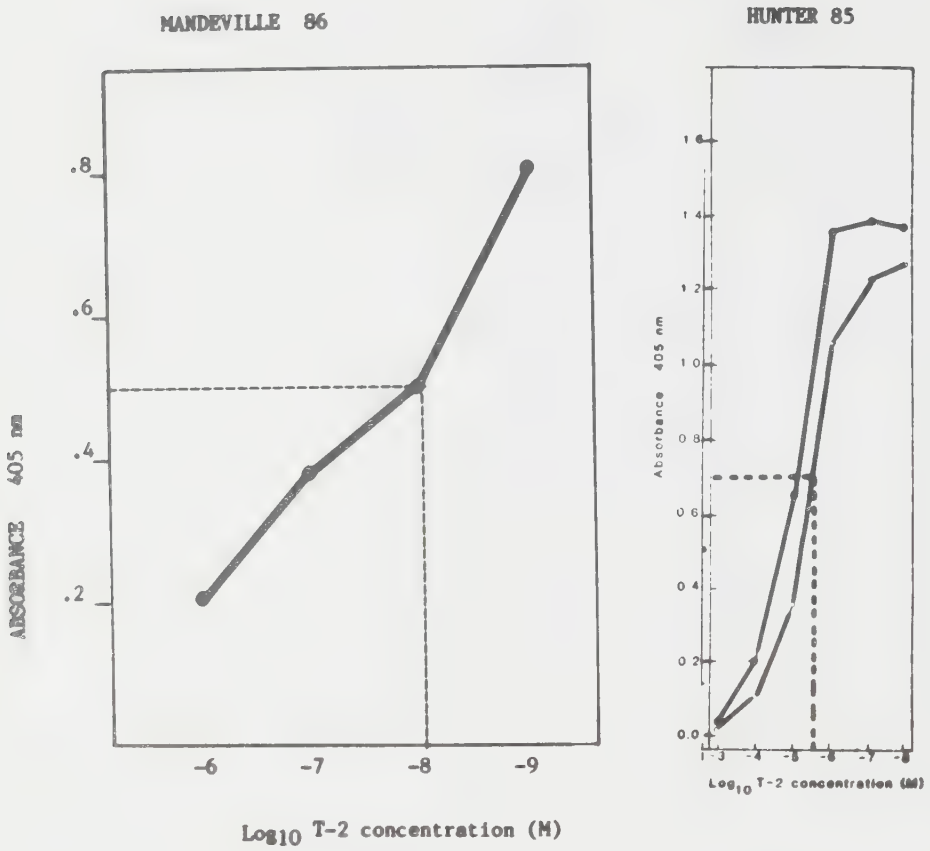
Rabbit T-2 Antisera	Protein Conc. (mg/ml)	Quantity Available	Dilution Used	No of Assays
5154-A*	6.54	33 ml	10^{-4}	330 000
4205-A	6.43	40 ml	10^{-4}	400 000
4204-A	5.50	5 ml	10^{-4}	50 000
5153-64*	- -	40 ml	- -	- -
5154 C	- -	10 ml	- -	- -
4205 C	_ _	10 ml	- -	- -

5154-A Absorbed antibodies

5153-64 Native antisera to be purified if needed.

FIGURE 12

COMPARISON OF CELISA WITH PUBLISHED DATA BY HUNTER (10)



50% inhibition = 2.1×10^{-8} M

= 10^{-6} M

CONCLUSIONS AND RECOMMENDATIONS

This report provides a detailed analysis of the work performed, outlines the problems encountered and some solutions to these problems. It should be understood that the TSK/T-2 kit is only a "demonstrator" and that a number of different problems should be addressed in order to establish the performance of this kit and to increase its reliability and specificity.

The following issues have been identified for consideration in the event further work might be contemplated.

A. First are some practical points to increase the performance of the TSK/T-2 kit:

   (a) The possibility of avoiding refrigeration altogether: peroxidase and H_2O_2 now have to remain at 3 to 10°C during transportation. It will be necessary to test a number of different peroxidases available on the market in order to select the one that is most suitable for lyophilization without losing its properties.

   (b) The possibility of further increasing the number of sera that could be tested in each of the plates and the number of total plates that could be included in each of the portable kits.

B. The second issue is the problem of sensitivity. It is possible to add a portable ELISA reader to this kit. Included as Appendix D are the specifications of certain fully automated readers (that can also be purchased in a custom case), their costs and descriptions.

The addition of an ELISA reader to the kit would combine higher accuracy (quantitative results compared to semi-quantitative results that can only be given by naked-eye examination) and very fast reading (60 seconds for a 96 well microtiter plate). It would also provide a printout that could be kept as a hard copy record.

C. The third issue addresses the problems of specificity. It would be necessary to study cross-reactivity with other trichothecene mycotoxins like DON, DAS and HT-2. IAF still holds 25 mg of HT-2, 27 mg of DAS and 100 mg of DON (provided by Dr. H. Cohen) that could be used in these studies.

D. The final issue involves further work to determine/confirm the accuracy of the technique. For example, this could involve a double blind study of T-2 samples which could be prepared by another laboratory. One could then compare the accuracy of our screening technique with that performed by TLC or GC in a laboratory setting.

E. Finally, it would be important to study the influence of several factors in the sera on the performance of TSK/T-2 kits:

- age, sex and race
- effect of blood hemolysis (and production of switch enzymes)
- effect of higher (40°C) and lower temperatures (0°C)
- effect of different physiological conditions: pregnancy, smoking

- effect of pathological conditions - hepatitis, cancer, high blood pressure, etc.
- drug interferences

IAF is very well equipped to perform this work and has established important contacts with major university hospitals within the Montreal area to be able to study most of the issues presented in this brief summary.

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APPENDIX A

GLOSSARY OF TERMS

A_{405}	Absorbance at 405 nm as read by spectrophotometer
ABTS-H_2O_2	2,2'-Azino-di(3-ethylbenzthiazoline sulfonate) - hydrogen Peroxide
Anti-T-2	Antibodies against T-2
BCG	Bacillus Calmette Guerin
BSA	Bovine Serum Albumin
CELISA	Competitive Enzyme Linked Immunosorbant Assay
CNBr	Cyanogen Bromide
CPM	Counts per Minutes
DAS	Diacetoxyscirpenol
DMF	Dimethylformamide
EDC	1-(3-dimethylaminopropyl)-3-ethyl carbodiimide HCl.
ELISA	Enzyme Linked Immunosorbant Assay
ETOH	Ethanol
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
HFBI	Heptafluorobutyrylimidazole
MAs	Monoclonal Antibodies
N2	Liquid Nitrogen
PBS	Phosphate Buffer Solution
POD	Peroxidase
PVP-40	Polyvinylpyrolidone
RIA	Radioimmunoassay
SDS	Sodium Dodecyl Sulfate
T-2 Toxin	3-hydroxy-4, 15-diacetoxy-8-(3-methyl-butyryloxy) 12, 13-epoxytrichothec-9-ene
^{3}H-T-2	Radioactive (tritium-labelled) T-2
THF	Tetrahydrofuran
v/v	Volume to Volume
w/v	Weight to Volume

APPENDIX B

PRODUCTION OF ANTI-T-2 MONOCLONAL ANTIBODIES

HYBRIDOMA PREPARATION

A series of fusions were done over several months with various standard immunization regimens (4,5), but no hybridomas secreting antibodies anti T-2 were obtained.

a. Animals and Immunization protocols:

Eight female BALB/c mice (20 g, body weight) were used for the production of monoclonal antibodies (MAs). They were immunized from 5 to 8 times using Hapten-Conjugate/FIA mixture (100 ul) containing approximately 1.4×10^{-7} mM BSA per injection or 34.4 ug per kg of body weight. As shown in Fig. 12 (p. 40), animals were injected from 6 to 8 times over the period of the study.

Animals were regularly bled from the tail and antibody development checked. Fig. 13 shows that animals produced low level of antibodies in their sera when monitored by RIA. The level of antibody production was also compared to that produced in rabbit's sera (Fig. 14) using RIA.

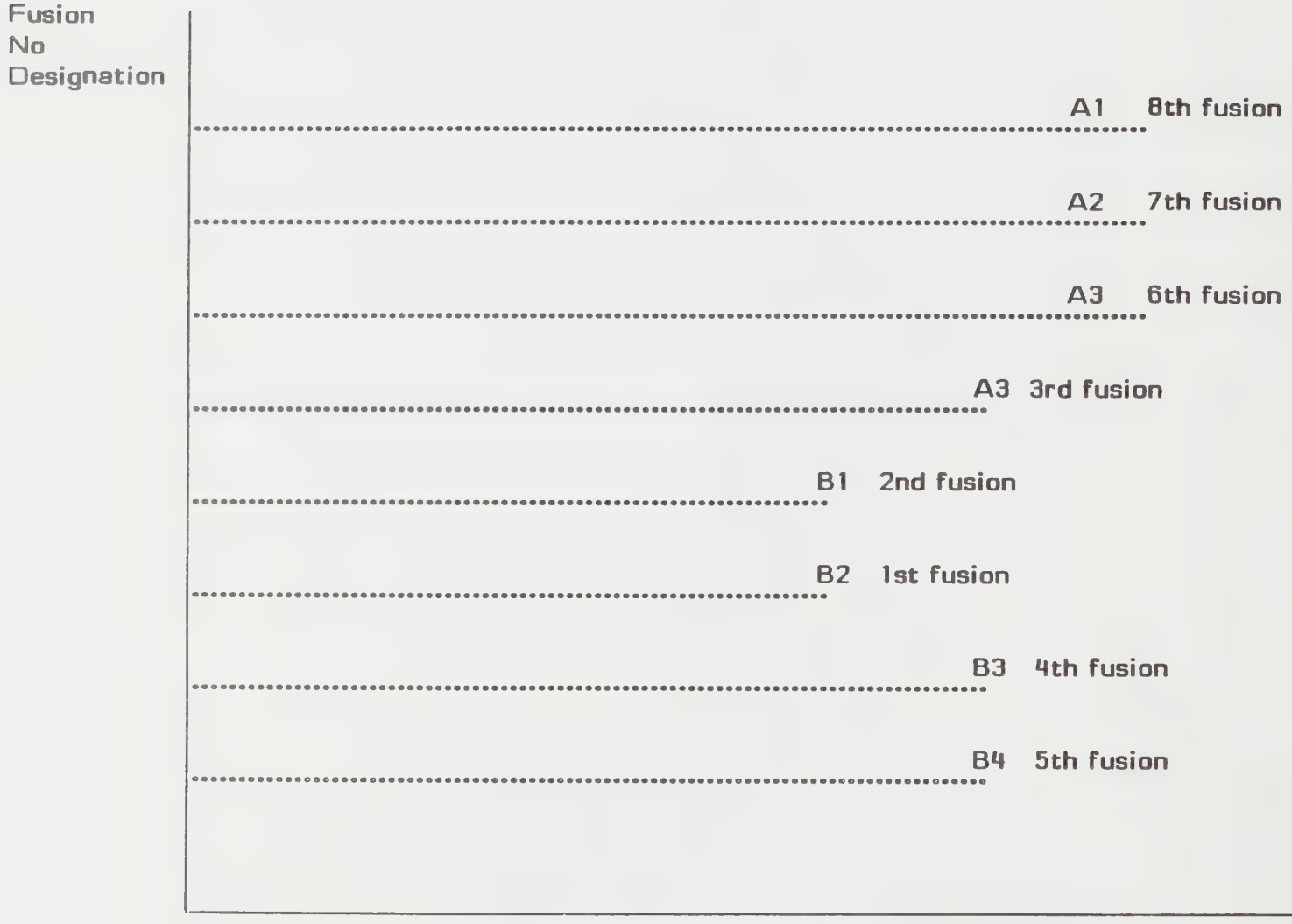
b. Fusions:

Animals were sacrificed at regular intervals and spleen cells were fused to myeloma cells (NS/1). Eight different fusions were carried out in the hybridoma service. In all fusions, hybrids were detected for 1 to 2 weeks. However, all cells died in culture. Fusion efficiency (number of wells with hybrids per number of wells seeded) was always very low; less than 10% and often less than 1%.

FIGURE 13

Mice Immunization Protocol Using T-2-BSA

Group A – T-2-BSA (Sigma)
Group B – T-2-BSA (Cohen)



Day	D1	D15	D30	D49	D72	D106	D120	D150
	30.04	14.05	29.05	17.06	10.07	13.08	27.08	26.09
Immunization No.	1st	2nd	3rd	4th	5th	6th	7th	8th

FIGURE 14

RIA Antibody Titration

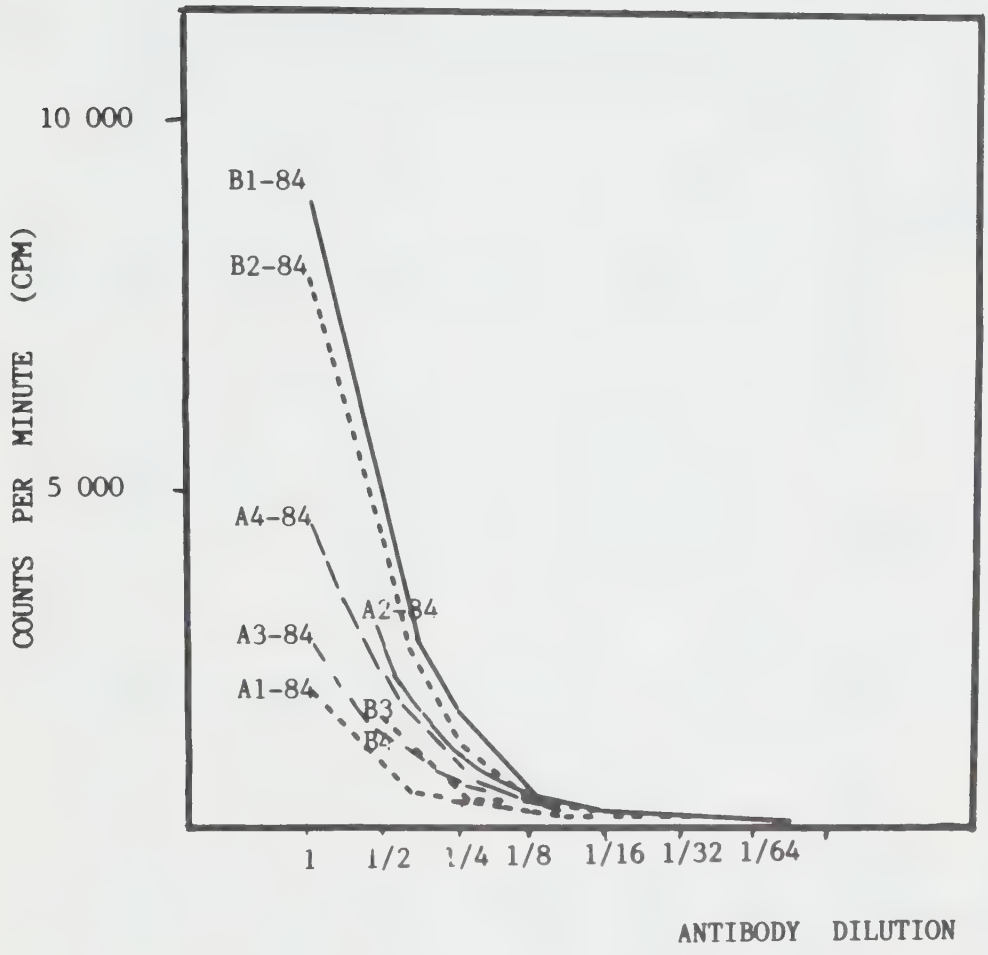
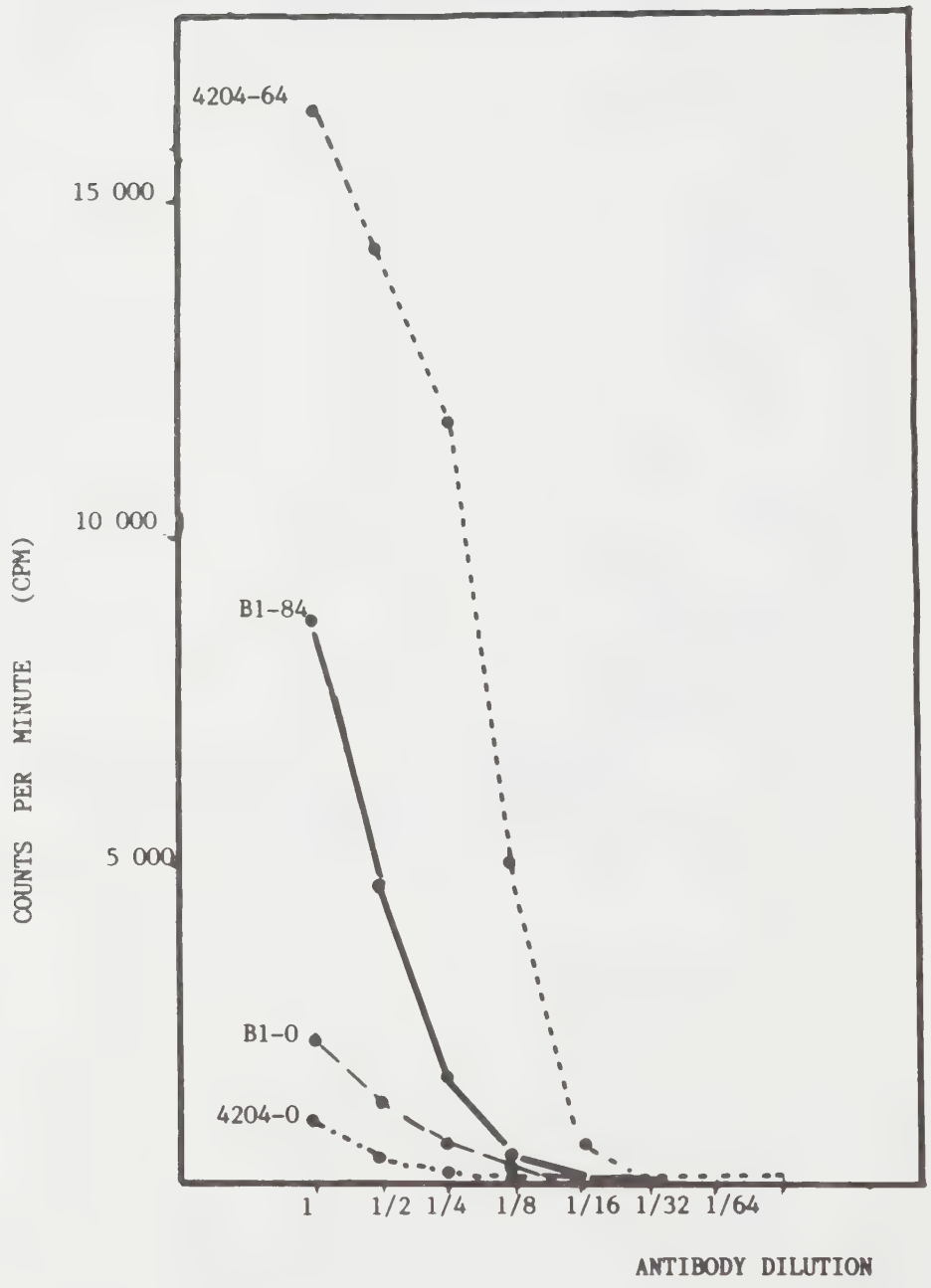


FIGURE 15

Comparison of antibody level in rabbits and mice



CYTOXICITY OF T-2 ON CELL LINES:

In order to investigate the cytoxicity of T-2 on cell lines of human and animal origin, several studies were performed (Tables 1 to 8). Tables 1 to 3 illustrate the toxicity on spleen cells cultured for 24 hrs with different concentrations of T-2 toxin; at 1 ug/ml, T-2 toxin was toxic for splenic cells of BALB/c mice after an incubation of 24 hrs.

T-2 cytotoxicity was also studied on cultured cell lines. We found that the toxic dose for each of the different cell line was:

Hela	0.05 ug/ml	(Table 6)
HBL-100	10 ng/ml	(Table 7)
BT-20	5 ng/ml	(Table 8).

However, for VERO cells even 0.1 ug/ml of T-2 was not toxic (Tables 4 and 5).

We concluded that T-2 cytotoxic activity varies from one cell line to another and could be one of the causes of the death of hybridoma cells as observed in all of our fusion experiments. Another explanation for this low cytotoxicity could be related to membrane transport problems that could have prevented us from observing a substantial toxicity on the cultured cell lines, but was encountered in our fusions.

TABLE 1

REPORT OF TOXICITY OF T-2 TOXIN

DATE:	May 6, 1985
CONCENTRATION OF TOXIN:	3 mg/ml
DILUTION:	10 ug/ml - 100 ug/ml
SOLVENT:	DMSO
CELLS:	Spleen cells of BALB/c mouse
READOUT:	24 hrs

DILUTION (ug/ml)	VIABILITY (%)
100	63
75	65
66	63
50	59
33	40.75
25	59
10	73

CONTROL: 82.5%

CONCLUSION

Toxicity at 10 ug/ml or less

TABLE 2

REPORT OF TOXICITY OF T-2 TOXIN

DATE:	April 24, 1985
CONCENTRATION OF SUBSTANCE:	0.1 ug/ml
DILUTION:	10 ug/ml - 10 ng/ml
SOLVENT:	Methanol
CELLS:	Spleen cells of BALB/c mouse
READOUT:	24 hrs

DILUTIONS (ug/ml)	VIABILITY (%)
10	66
1	65
0.1	74
0.01	79
CONTROL:	81

CONCLUSION:

Toxicity at 1 ug/ml

TABLE 3

REPORT OF TOXICITY OF T-2 TOXIN

DATE:	April 1, 1985
CONCENTRATION OF SUBSTANCE:	0.1 ug/ml
DILUTION:	10 ng/ml - 10 pg/ml
SOLVENT:	DMSO
CELLS:	Spleen cells of BALB/c mouse
READOUT:	24 hrs

DILUTIONS (ng/ml)	VIABILITY (%)
10	82
1	87
0.1	80
0.01	74
CONTROL	80

CONCLUSION

No toxicity after 24 hours

TABLE 4

REPORT OF TOXICITY OF T-2 TOXIN

DATE:	June 25, 1985
CONCENTRATION OF TOXIN:	1000 ug/ml
DILUTIONS:	0.1 ug/ml - 0.005 ug/ml
SOLVENT:	DMSO
CELLS:	VERO
READOUT:	24 hrs

DILUTIONS (ug/ml)	CYTOTOXICITY (%)
0.1	0
0.05	0
0.01	0
0.005	0

CONCLUSION

No inhibition of growth at 0.1 ug/ml

TABLE 5

REPORT OF TOXICITY OF T-2 TOXIN

DATE:	June 12, 1985
CONCENTRATION OF TOXIN:	1200 ug/ml
DILUTIONS:	0.1 ug/ml - 0.001 ug/ml
SOLVENT:	DMSO
CELLS:	VERO
READOUT:	24 hrs

DILUTIONS (ug/ml)	CYTOTOXICITY (%)
0.1	0
0.05	0
0.01	0
0.005	0
0.001	0

CONCLUSION

No inhibition of growth

TABLE 6

REPORT OF TOXICITY OF T-2 TOXIN

DATE:	June 12, 1985
CONCENTRATION OF SUBSTANCE:	1000 ug/ml
DILUTIONS:	0.1 ug/ml - 0.001 ug/ml
SOLVENT:	DMSO
CELLS:	HeLa
READOUT:	24 hrs

DILUTIONS (ug/ml)	CYTOTOXICITY (%)
0.1	63
0.05	50.5
0.01	25.5
0.005	19.25
0.001	0

CONCLUSION

50% inhibition at 0.05 ug/ml

TABLE 7

REPORT OF TOXICITY OF T-2 TOXIN

DATE:	March 26, 1985
CONCENTRATION OF SUBSTANCE:	1000 ug/ml
DILUTION:	0.1 ug/ml - 0.005 ug/ml
SOLVENT:	DMSO
CELLS:	HBL-100
READOUT:	48 hrs

DILUTIONS (ug/ml)	CYTOTOXICITY (%)
0.1	100
0.05	100
0.020	100
0.010	50
0.005	40

CONCLUSION

50% cytotoxicity at 0.01 ug/ml

TABLE 8

REPORT OF TOXICITY OF T-2 TOXIN

DATE:	March 26, 1985
CONCENTRATION OF SUBSTANCE:	1000 ug/ml
DILUTION:	0.1 ug/ml - 0.005 ug/ml
SOLVENT:	DMSO
CELLS:	BT-20
READOUT:	48 hrs

DILUTIONS (ug/ml)	CYTOTOXICITY (%)
0.1	100
0.05	100
0.020	100
0.010	75
0.005	50

CONCLUSION

50% cytotoxicity at 0.005 ug/ml

APPENDIX C

THE TSK/T-2 KIT

WARNING FOR USERS:

1. Do not pipette reagents and samples by mouth at any time.
2. A laboratory coat or other suitable protective clothing such as disposable plastic gloves (provided in the kit) should be used throughout the testing procedure.
3. All spillage should be immediately wiped up thoroughly and contaminated material disposed of in a suitable manner: either by soaking for 2 hours (or more) in javex (5% solution or better) or by burning.

STORAGE INFORMATION

1. H_2O_2 and peroxidase-labelled goat anti-rabbit IgG should be refrigerated (2°C to 8°C). All the other reagents can be stored at room temperature (20°C to 30°C). Avoid freezing of reagents.
2. All reagents must be brought to room temperature (20° to 30°C) before being used.

SPECIMEN COLLECTION AND HANDLING:

Serum and plasma may be tested with TSK/T-2. Specimens should be clear and non-hemolyzed whenever possible. Plasma should be fresh, not frozen and thawed. To separate plasma let blood specimen stand at room temperature (20°C to 30°C) for 2 to 4 hours. If kept at 4°C, let stand for 16 hours (room temperature accelerates the separation of red blood cells from plasma). If the test is to be run within 24 hrs after collection, the specimen should be kept refrigerated at all times.

This new kit, called TSK/T-2, allows the rapid detection of T-2 toxins in human serum and plasma. The CELISA technique itself is a competition between T-2 on the bottom of the wells and T-2 in the test serum specimen for binding to anti-T-2 antibody. It is based essentially on a color change. As illustrated in the color chart (photograph provided) a dark green colour indicates no T-2 in the plasma sample (or, more properly, that any T-2 present, is below the detection threshold of the kit); if the color of the assay changes to lighter green, this indicates the presence of T-2 in the specimen. The assay is easy to perform with no extraction procedures involved. It can be performed by any paramedical personnel and the result evaluated by naked-eye examination. It does not therefore need any elaborate apparatus.

SUMMARY AND EXPLANATION OF THE TEST:

The TSK/T-2 system is a CELISA (or Competitive ELISA) based on the "sandwich" principle. The multiwell plastic plate is pre-coated with T-2 toxin. To perform the test, serum specimens, standards and controls are incubated at room temperature with anti-T-2 antibodies. During this incubation the T-2 present (in the preparations to be tested) binds to the anti-T-2 antibodies (primary antibody), thus forming antigen-antibody complexes. Aliquots of these mixtures are then added to the T-2 coated wells to allow free antibodies to bind to T-2 on the plate. Unbound materials are removed by repeated washings of the wells. Goat anti-rabbit IgG (secondary antibody) labelled with peroxidase is then added to the well to reveal the presence of the antigen-antibody complexes. The enzyme-substrate is finally added to reveal the presence of fixed enzyme to the antigen-antibody complexes. Dark green indicates no T-2 in the plasma sample while paler shades of green indicate the presence of T-2.

CONTENTS OF THE TSK/T-2 KIT:

1. One microtiter plate pre-coated with T-2.
2. Reagents include:
   a. Anti-T-2 antibody (rabbit polyclonal) lyophilized + 1 ml of deionized water + 8.75 ml of washing buffer (vial S).
   b. Goat anti-rabbit IgG labeled with peroxidase (affinity purified) + 12.5 ml dilution buffer (vial D).
   c. Positive control (200 ug of T-2 toxin) in a lyophilized form + ethanol (95%) + 1 ml of washing buffer (vial T).
   d. Substrate including ABTS in powder form and 1 ml of H_2O_2 (30%) + 1 ml of substrate buffer (vial F).
   e. Stopping reagent for the peroxidase reaction (vial P).
   f. Concentrated (20x) washing buffer (vial W) to add to large bottle of H_2O (190 ml).
3. Additional items include:
   a. A pair of plastic disposable gloves
   b. Precision pipettes: (2 x 5 ml + 1 ml + 2 x 10 ul).
   c. Test tubes (18 snap-capped tubes of 5 ml each).

Although the materials supplied can be used to perform more assays, the two "demonstrator" kits provided will allow the testing of only 5 sera samples. After reconstitution of the solutions and performance of the tests, any remaining reagents or solutions should be discarded by decontamination in javex (5% solution or better) or by burning. Do not store any reconstituted materials as it could affect the performance of the kit.

PREPARATION OF WORKING SOLUTIONS:

1. 1 vial of rabbit anti-T-2 serum (lyophilized). To reconstitute add 0.1 ml of deionized water. Immediately before use, add one drop of reconstituted rabbit anti-T-2 serum to the 8.75 ml solution of washing buffer (vial S). Vial S is now considered as the working solution.
2. 1 vial of T-2 Toxin (200 ug). Add one drop of ethanol to dilute the powder, then add 1 ml of washing buffer (vial T) to tube labelled T-2 Toxin. Vial labelled T-2 Toxin is now considered as the working solution.
3. 1 vial of peroxidase-labelled goat anti-rabbit IgG (affinity purified). To dilute the peroxidase take 1 ml of dilution buffer (vial D) and thoroughly rinse the peroxidase vial. Then, add this mixture to vial D. Vial D is now ready for use.
4. Vial W contains concentrated Washing Buffer (20x) to be added to the 190 ml of deionized water. This solution now becomes the diluted Washing Buffer.
5. 1 vial of ABTS substrate powder (vial F). Dissolve by adding 10 ml of substrate buffer. Immediately before use, add 10 ul of 30% H_2O_2 (vial H) to the ABTS solution (vial F). Vial F is now the working solution.
6. Vial P contains stopping reagent for peroxidase reaction (5% of SDS in a powder form). Dissolve this powder in 20 ml of the diluted washing buffer.

ASSAY PROCEDURE

Bring specimens and reagents to room temperature (20° to 30°C) before use. To avoid cross-contamination, use a clean disposable pipette for each of the serum samples and for each of the standard solutions.

Step No. 1: Preparation of T-2 Standard Solutions:

- Pipette accurately 0.9 ml of the diluted Washing Buffer into the 4 snap capped tubes pre-labeled: B1, B2, B3 and B4.
- Serially dilute T-2 Toxin by adding 3 drops (with a new disposable plastic transfer pipette) of T-2 Toxin from reconstituted vial labelled T-2 Toxin to tube B1. Mix well (by simple hand agitation). Now take 3 drops from tube labeled B1 and place in tube labeled B2 and mix well.
- Repeat the dilution step by transferring 3 drops of B2 to B3, and then 3 drops from B3 to B4.
- Tubes B1, B2, B3 and B4 constitute successive 10 fold dilutions (1, 1/10, 1/100 and 1/1000 respectively) of the T-2 standard solution and will be used as positive controls for this assay.
- Starting with the more diluted preparation (B4) and using only one pipette, transfer 0.5 ml from each of these tubes (B4, B3, B2 and B1) to tubes SB4, SB3, SB2 and SB1 respectively. To each tube add 0.5 ml of reconstituted rabbit anti-T-2 serum (vial S).

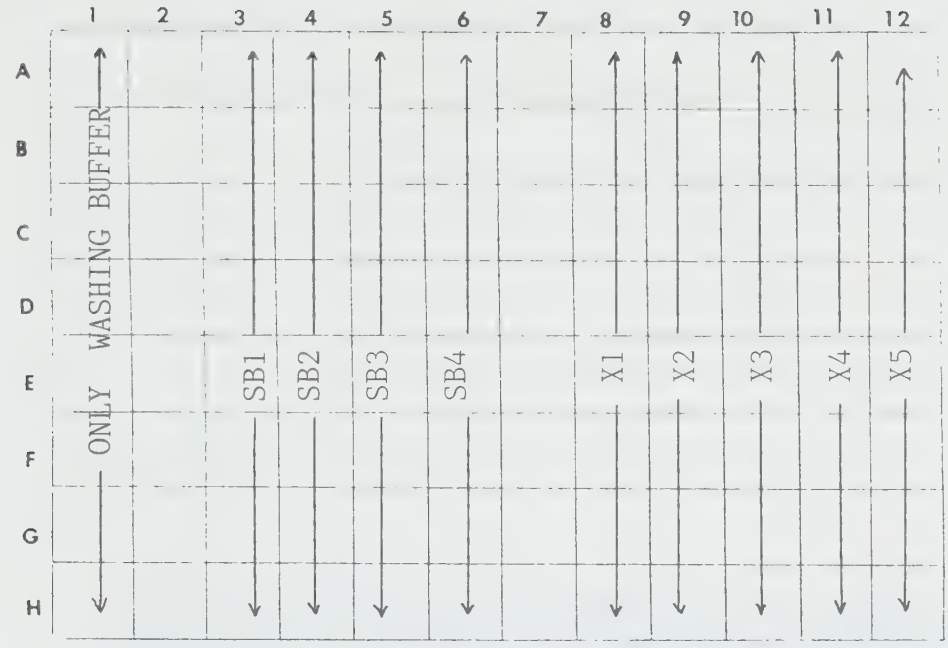
Step No. 2: Preparation of Serum Material:

As shown in the diagram below, the kit (as presently constituted) allows for testing only 5 different serum samples.

- Collect samples of blood (2 to 5 ml from each individual to be investigated) in unlabelled snap-capped tubes. Let stand at room temperature for 2 to 4 hours or at 4°C for 16 hours. A clot will form and the serum will separate (label each tube from 1 to 5).
- Put 0.5 ml of each of the serum samples in each of the corresponding tubes pre-labelled X1, X2, X3, X4 and X5.
- Then add 0.5 ml of diluted rabbit anti-T-2 serum (vial S) to each of these tubes (X1 to X5).
- Replace cap and mix well by simple hand agitation.

Step No. 3: Pre-Incubation:

Incubate standards (tubes labelled SB in Step No. 1) and serum material (tubes labelled X and prepared in step No. 2) at room temperature (20°C to 30°C) for 2 hrs.



T-2 controls Serum samples

REFERENCE CHART FOR THE ADDITION OF TEST SERUM AND STANDARDS

STEP No. 4: Addition of Test Serum and Standards to Wells:

- As illustrated in the diagram above and using a clean pipette, wash the microtiter plate (3x) by adding (6 drops per well) of Washing Buffer. Discard the used Washing Buffer in a Javex container.

- Add the pre-incubated materials (samples and standards prepared in Step No. 2 and No. 3) to the appropriate rows (3 drops per well). Row 3 to 6 (A-H) contain SB1 to SB4; Row 8-12 (A-H) should now contain X1 to X5.

- Row No. 1 (A-H) is always used for background control and only Washing Buffer is added to each of the wells in this row (3 drops per well).
- Rows No. 2 (A-H) and No. 7 (A-H) are kept empty.
- Incubate the microtiter plate at room temperature (20°C to 30°C) for 2 hrs.
- Wash each microtiter plate well (3x) with 6 drops per well of Washing Buffer.

Step No. 5: Addition of enzyme and its substrate:

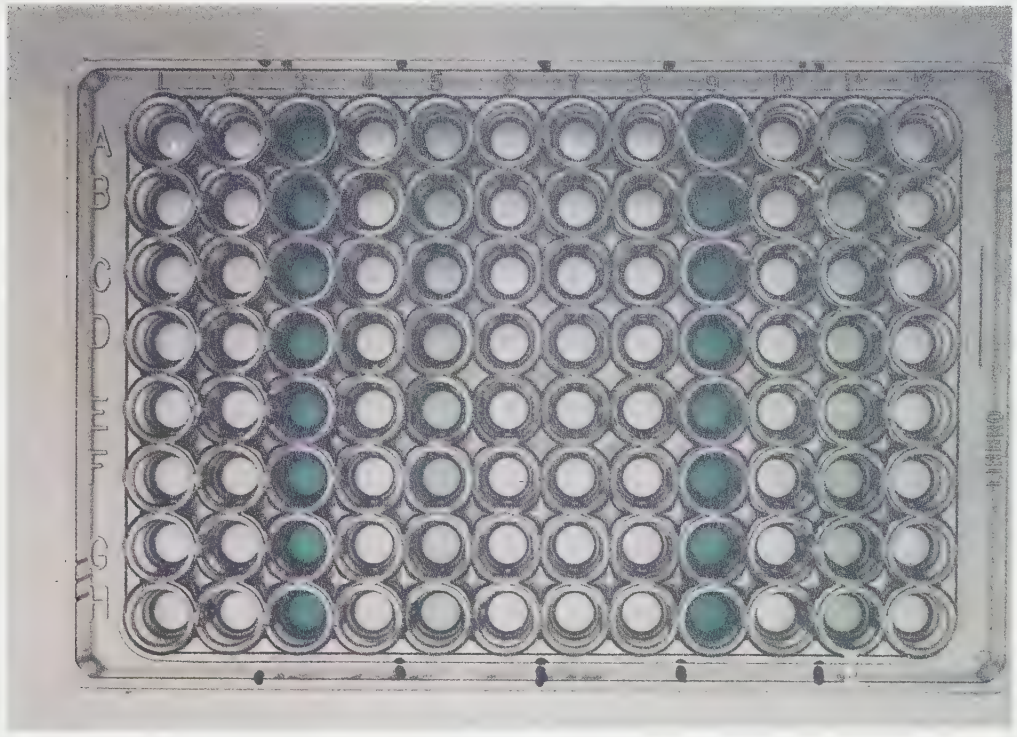
- Add 3 drops of reconstituted peroxidase-labelled goat anti-rabbit IgG antibody to each of the microtiter plate wells (vial D).
- Incubate at room temperature (20°C to 30°C) for 2 hrs.
- Wash each microtiter plate well (3x) with 6 drops per well of Washing Buffer.
- Add 3 drops of freshly prepared ABTS-H_2O_2 substrate (vial F) to each well and incubate at room temperature (20°C to 30°C) for 10 to 20 minutes or until standards (Rows 3-6 (A-H)approximate the colours appearing in the reference colour photograph provided.

Step No. 6: Stopping the reaction:

Add 1 drop of stopping reagent (vial P) to each microtiter plate well to stabilize the peroxidase reaction and to stop further darkening of the color.

INTERPRETATION OF THE RESULTS

For a semi-quantitative evaluation of the results, observe the change in color and compare to chart (illustrated in reference chart below). A dark green color means absence of T-2 in the serum SAMPLE (Rows no 3 and 9 or, more properly, that if any T-2 is present, it is below the detection threshold of the kit i.e., below 2.1 X 10^{-8} M). Paler shades of green denote the presence of T-2 in the serum sample (Rows no 5, 7 and 11). For a more quantitative evaluation, absorbance at 405 nm can be measured using a spectrophotometer.



REFERENCE CHART TO BE USED FOR INDICATION OF COLOR CHANGE

Rows 2, 4, 6, 8, 10 and 12 are empty.

Row no 1 contains only washing buffer (negative color control).

Rows no 3, 5, 7, 9 and 11 contain different concentrations of T-2 to illustrate gradation of color. Rows no 3 and 9 illustrate that the preparation in testing does not contain any T-2 (or below 2 X 10^{-8}M); row no 7 that the preparation contains a large amount of T-2 (2x10^{-6}M or above); and rows no 5, 7 and 11 illustrate that the preparation in testing contain concentration in between 2 X10-^{6}M and 2x10-^{8}M.

REMINDER

Whereas a portable spectophotometer could provide accurate estimates of the quantity of T-2 in the serum samples based on color change, this is not the case for purely visual analysis. In the latter instance, all that is important is that this test indicates the presence of T-2, since this is only a screening kit. The samples should then be appropriately packaged, stored and transported to a laboratory for subsequent analysis.

APPENDIX D

PORTABLE ELISA READER

Model 2550 EIA Reader

BIO-RAD

Reads 96 Wells in Less Than 1 Minute

Microprocessor and fiber optic technology combine in the Bio-Rad Model 2550 EIA reader to create a photometer that automatically reads, with blanking and hard copy printout, a 96 well microtitration plate in less than 60 seconds. The Model 2550 incorporates many of the features found on more expensive photometers, yet it is the first fully automatic microtitration plate reader priced so economically that any laboratory can easily afford to own one.

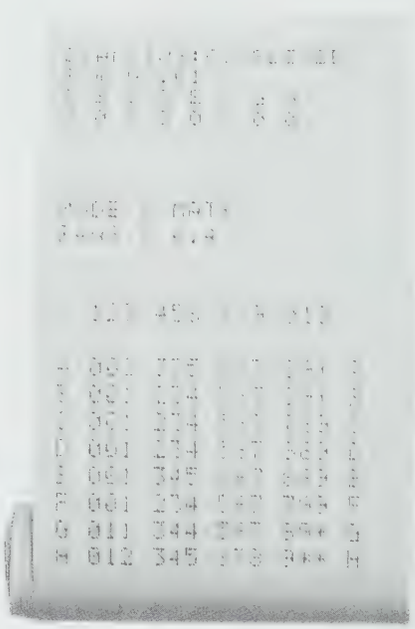
Enzyme immunoassays are rapidly replacing many other methods for detecting or quantitating substances with important biological or pharmacological properties. The new Model 2550 EIA reader has the speed, sensitivity and versatility required for these applications. Its unique, user friendly, membrane control pad makes function entry fast and simple. A digital absorbance-range display (matrix mode) and plate-position indicator, as well as an operator error indicator and an absorbance overflow indicator, guide you during instrument operation. A convenient step control function allows you to advance the plate manually, if automatic reading is not desired.

State-of-the-Art Fiber Optics

The EIA reader, which operates as a vertical-pathlength photometer, measures the absorbances of the contents of eight wells, or one row, of a 96 well microtitration plate simultaneously. Using state-of-the-art fiber optics, light is passed upward through the bottom of the microtitration plate, which acts as an optical window, then through the surface of the solution to a bank of eight photo-detectors. This method of measuring absorbance eliminates variability caused by reagent evaporation, because the absorbance measurement is based only on the absolute amount of absorbing material present, and not on its concentration. This proven, sensitive, and widely accepted measuring method allows rapid direct reading of the samples in the microtitration plate. Because the contents of the wells are not aspirated during reading, the results can be verified by re-reading the plate, if desired.

Versatility

The Model 2550 is an extremely versatile instrument. Four high quality, narrow bandwidth interference filters, 405, 414, 450 and 492 nm, are supplied as standard equipment. Other, optional, filters permit wavelength measurement over the range of 380 to 700 nm. The reader has two measuring modes, absorbance mode or matrix mode. The absorbance mode provides a direct hard-copy printout of the absorbance values to three decimal places, identifying corresponding column and row positions on the printout. The absorbance mode can process readings of up to at least 1.5 absorbance units with guaranteed linearity. The matrix mode lets you preselect an absorbance range, and automatically divide it into ten equal segments. The microprocessor assigns a value from 0–9 to each segment, and assay absorbance readings are cataloged into the appropriate range segment. All values are printed out in an 8 × 12 matrix that corresponds exactly to the layout of the 96 well microtitration plate. Absorbance values up to 2.9 absorbance units can be processed using the matrix mode. This mode is particularly useful for end-point determinations or quick screening of assays. These two operating modes, together with the wide selection of filters, provide the flexibility needed for performing enzyme-linked immunosorbent assays (ELISA), monoclonal antibody screening by ELISA, agglutination/haemagglutination assays, complement fixation, antibiotic sensitivity testing (MIC), protein determinations and several other assay procedures requiring colorimetric measurement.



Microprocessor Control

All data processing and operating functions are controlled by the EIA reader's central microprocessor (CPU). This microprocessor control insures reliability, speed, and simplicity of operation. Because the instrument can read a microtitration plate in less than 60 seconds, any effects arising from reagent deterioration are eliminated. The microprocessor can also be interfaced with larger computers using the optional Bio-Rad serial (RS-232-C) or parallel (IEEE 488) interface devices. This makes the EIA reader ideal for high volume laboratories that require sophisticated data manipulation.

Complete Data Handling with Apple® Computer Based System

Bio-Rad's EIA reader and Apple computer system offer all the computing power necessary for even the most demanding laboratories. Together they provide the experienced Immunologist/Programmer with a matched, high performance noniso-topic assay system.

Specifications*

Operating Modes:	**Absorbance** – Direct printout to three decimal places with corresponding column and row positions.
	Matrix – Numerical printout in 8 × 12 matrix.
Measurement Range:	Linear – 0 to 1.5 AU absorbance mode. Will process up to 2.9 AU matrix mode.
Light Source:	8V/50W Tungsten Halogen Lamp.
Wavelength Range:	380 nm to 700 nm
Wavelength Selection:	High quality interference filters.**
Warm up Time:	15 minutes
Measurement Time:	Less than 60 seconds, with a hard copy printout (typical).
Without Blanking:	57 seconds start to printout (typical)
With Blanking:	62 seconds blanking to printout (typical)
Stability (15 minute warm up):	Drift ⩽ 0.006 OD/hour over eight hours (typical).
Reproducibility (15 minute warm up):	⩽ ± 0.008 OD channel to channel (typical).
Line Voltages:	90–132 VAC, 50/60 Hz 198–264 VAC, 50/60 Hz
Power Consumption:	100 VA
AC Line Cord:	Detachable, country specific, line cords.
Weight:	8.5 kg
Size:	40 cm (W) × 13 cm (H) × 30.5 cm (D)
Memory Protection for Blank Function:	Retains blank value (zero value) up to 48 hours after line power interruption.
Thermal Dot Matrix Printer:	Paper width: 60 mm Speed: 1.8 lines per second Two rolls of paper are supplied.
Computer Interfacing (Optional):	Serial Mode: RS-232-C Parallel Mode: IEEE 488
	All Model 2550 EIA Readers are equipped with required pin connector for connection to optional interface device. Connection cables are optional.

*Specifications are subject to change without notice.

**Standard bandwidth 405, 414, 450 and 492 nm filters supplied as standard equipment. Other filters covering wavelengths from 380–700 nm are available.

Affinity Purified Antibodies

Bio-Rad now offers an extensive line of affinity purified antibodies and enzyme-antibody conjugates suitable for use in all microtitration EIA systems. Affinity purified antibodies conjugated to the enzymes horseradish peroxidase and alkaline phosphatase are available. Conjugated antibodies of goat origin are directed against human, rat, swine, rabbit and mouse immunoglobulins. Conjugated antibodies of rabbit origin are directed against sheep and goat immunoglobulins. Additionally, Bio-Rad offers affinity purified unconjugated antibodies of goat origin that are directed against human IgA, or IgM, or IgG, or IgE, rabbit immunoglobulin and mouse immunoglobulin.

Affinity purified antibodies are isolated from crude antisera by passage over affinity columns containing immobilized antigen. Affinity purification removes most nonspecific antibodies, other serum proteins, and endogenous enzymes that often interfere in various assay procedures. Thus, the use of affinity purified antibodies:

- Is more cost effective because less reagent is required.
- Results in shorter assay times because the specific antibody is more concentrated due to affinity purification.
- Minimizes false positive reactions because most nonspecific antibodies and extraneous serum proteins have been removed.
- Increases sensitivity because background noise is reduced.

EIA Substrates for Alkaline Phosphatase and Horseradish Peroxidase Labeled Antibodies

Alkaline Phosphatase Substrate Kit

Bio-Rad's new Alkaline Phosphatase Substrate Kit is used in soluble EIA systems that employ alkaline phosphatase labeled antibodies. The kit contains 100 five milligram tablets of p-nitrophenyl phosphate, and 100 ml of 5X concentrate diethanolamine buffer. This is enough to prepare 500 ml of substrate solution. In the presence of alkaline phosphatase the substrate solution develops an intense yellow color, measurable at 405 nm.

Horseradish Peroxidase Substrate Kit

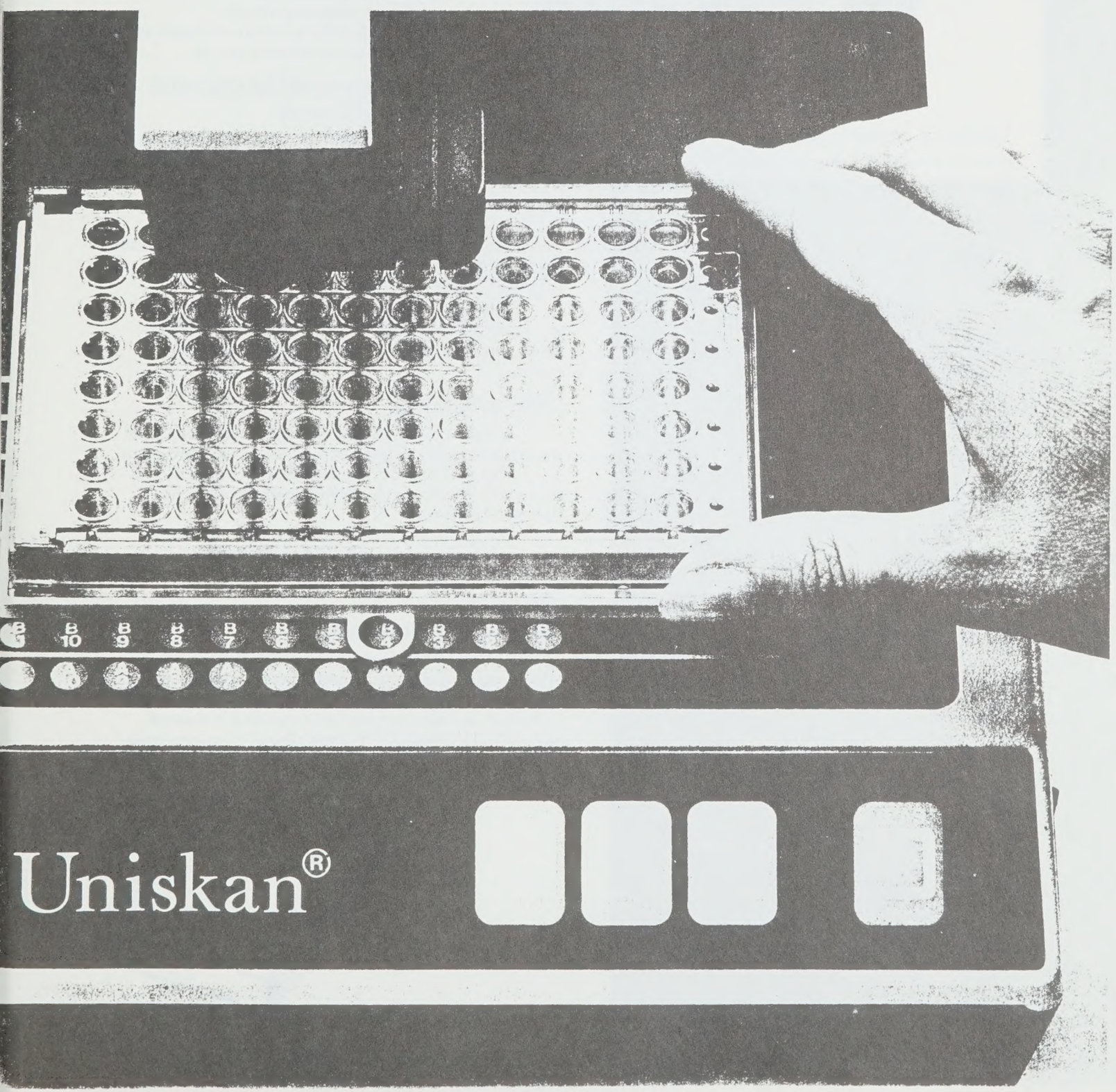
The new Horseradish Peroxidase Substrate Kit is used in soluble EIA systems that employ horseradish peroxidase (HRP) labeled antibodies. The kit contains two solutions, and is very easy to use. Solution A contains 2, 2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] in cacodylic acid buffer. Solution B contains hydrogen peroxide. The kit includes enough Solution A and Solution B to prepare 200 ml substrate solution. In the presence of HRP, the substrate solution develops an intense blue-green color, measurable at 414 nm.

Pipets, Tips and Plates for ELISA

For rapid, convenient 8-well pipetting we recommend Costar Octapette® together with Bio-Rad's MTP-28 tips in totally enclosed autoclavable racks, plus Costar 96-well flat bottom EIA microtitration plates.

Uniskan

expands your capacity for photometric measurements



Uniskan is compact. All accessories required in measurement come in the handsome case supplied with Uniskan.

There has been a gap in photometric measuring techniques and a need for an easy–to–use portable photometer.

Now the gap has been closed.

Uniskan is just the right solution for small laboratories in need of good photometric measurement results.

The versatile measuring head

Uniskan's versatility arises from the unique adjustable measuring head. You can adjust the height of the measuring head to fit the specifications of different measuring vessels.

Uniskan is battery-operated, so you can use it wherever you go.

Ideal for small laboratories and field work

With Uniskan small laboratories expand the range of their in–house capabilities to photometric measurements previously confined to large laboratories.

And for the first time, you can do photometric work in the field. You can measure sensitive specimens on the spot and save the trouble of storing samples and shipping them to large labs.

Uniskan's versatility works the other way around, too. It increases the capabilities of your laboratory by functioning as a back–up unit to a larger analyzing system.

Unique features

Uniskan has a number of unique features to make your photometric measurements all the more convenient.

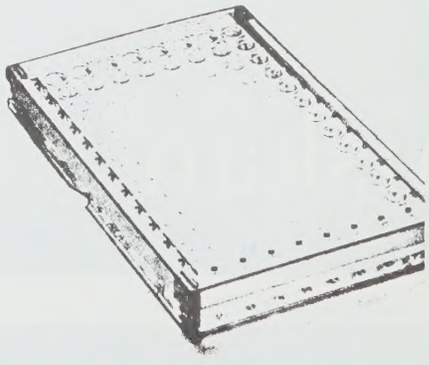
The pulsing lamp requires no warm–up time and guarantees ten times as many measurements as other equivalent devices.

The protected pushbuttons and self–testing programs assure you won't spoil your measurement.

And the built–in printer documents the correct data for you.

Wide application areas

Uniskan is designed to be used in various colorimetric and turbidimetric measurements typical to enzyme immunoassay (EIA) and microbiological work. A simple 340 nm filter and a temperature-controlled 9-channel cuvette block holder (37°C) add clinical chemistry capability.



Typical Uniskan applications involve Microplates, Microstrips and Cuvette Blocks.